

HETEROGENEITY WITHIN MACROPHAGE POPULATIONS:
A POSSIBLE ROLE FOR COLONY STIMULATING FACTORS

1988

FALK

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ABSTRACT

Title of Dissertation: Heterogeneity within Macrophage Populations: a Possible Role for Colony Stimulating Factors

Lydia A. Falk, Doctor of Philosophy, 1988

Dissertation directed by: Stefanie N. Vogel, Associate Professor, Department of Microbiology

These studies were undertaken in an attempt to elucidate the raison d'etre for the existence of two distinct Colony Stimulating Factors (CSFs) whose site of action is believed to be the same bone marrow progenitor and whose differentiated mononuclear "end cell" is presumed to be identical. Through a side by side comparison, we have explored this question of apparent cytokine redundancy by examining the responsiveness of murine bone marrow progenitors to highly purified or recombinant preparations of GM-CSF and CSF-1, in both soft agar and liquid cultures. Our findings show that the number of CSF-1-responsive progenitor cells which formed colonies in soft agar was approximately six-fold greater than the number of progenitors which responded to GM-CSF. However, upon stimulation of bone marrow progenitors in soft agar culture with both GM-CSF and CSF-1, we observed the development of a significant percentage of very large colonies (≥ 2 mm in diameter). These progenitors were present in the bone marrow of both untreated mice and in mice which had been administered the cytotoxic drug, 5-fluorouracil, consistent with descriptions of a primitive progenitor population which exhibits high proliferative potential (HPP-CFC). In addition, the macrophages which developed under the influence of either GM-CSF or CSF-1 in liquid culture were found to differ morphologically and functionally. Although CSF-1-derived macrophages were shown to be superior in their phagocytic capacities and the ability to resist viral infection, GM-CSF-derived macrophages exhibited very high Ia antigen expression, an augmented ability to induce antigen-specific T cell proliferation, and a greater potential for tumoricidal activity. These findings suggest that the acquisition of "higher order"

macrophage functions (i.e., typically associated with "highly activated" macrophages) may not require a differentiative progression which results in the retention of certain "lower order" functions. Our findings also imply that the heterogeneity observed among different populations of mature macrophages may reflect the effects of different species of colony stimulating factors on bone marrow progenitors and the emigration of selected populations from the bone marrow to peripheral sites.

HETEROGENEITY WITHIN MACROPHAGE POPULATIONS:
A POSSIBLE ROLE FOR COLONY STIMULATING FACTORS

by

Lydia A. Falk

Dissertation submitted to the Faculty of the Department of Microbiology
Graduate Program of the Uniformed Services University of the Health Sciences
in partial fulfillment of the requirements for the degree of
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To my loving husband, Rick, and to my mother and father, whose faith, love, and encouragement have been the mainstay of my existence.

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ABBREVIATIONS

ACAS, anchored cell analysis and sorting
AgNO₃, silver nitrate
ANOVA, analysis of variance
BCG, Bacillus Calmette Guerin
Bio-HT, hydroxylapatite
BMP, bone marrow progenitors
BSA, bovine serum albumin
But-LPS, butanol-extracted lipopolysaccharide
°C, degrees centigrade
cc, cubic centimeter
CC, cell control
cDNA, complimentary deoxyribonucleic acid
CFU, colony-forming unit
CFU-c, colony forming unit-culture
CFU-s, colony forming unit-spleen
Ci, curie
CPE, cytopathic effect
⁵¹Cr, chromium-51
CSF, Colony Stimulating Factor
CSF-1, macrophage colony stimulating factor
CO₂, carbon dioxide
cpm, counts per minute
D, day
DEAE, diethylaminoethyl

EBSS, Earle's Balanced Salt Solution

EDTA, ethylenediaminetetraacetic acid

ELISA, enzyme-linked immunosorbent assay

EMEM, Earle's Minimum Essential Medium

FCS, fetal calf serum

5-FU, 5-fluorouracil

G-CFC, granulocyte colony-forming cells

G-CSF, granulocyte colony-stimulating factor

g/l, grams per liter

GM-CFC, granulocyte-macrophage colony-forming cell

GS, goat serum

^3H , tritiated

HPLC, high performance liquid chromatography

HPP-CFC, high proliferative colony-forming cell

HSCM, human spleen cell conditioned medium

HU α CSF-1, human recombinant macrophage colony stimulating factor

HU α GM-CSF, human recombinant granulocyte-macrophage colony stimulating factor

Ia, I-region associated

IAA, iodoacetic acid

IFN- α/β , interferon-alpha/beta

IFN- γ , interferon-gamma

IgG, immunoglobulin isotype G

IL 1, interleukin 1

IL 2, interleukin 2

IL 3, interleukin 3

IL 4, interleukin 4

IU, international unit

kg, kilogram

KLH, keyhole limpet hemocyanin

LAP, lipid-A associated protein

LSD, least significant difference

LPS, lipopolysaccharide

LSM, lymphocyte separation medium

μ , microns

M, molar

ma, milliamps

MAF, macrophage activating factor

M-CFC, macrophage colony-forming cell

μ g, microgram

mg, milligram

MHC, major histocompatibility complex

ml, milliliter

mm, millimeter

mM, millimolar

M.O.I., multiplicity of infection

mRNA, messenger ribonucleic acid

NaCl, sodium chloride

NaOH, sodium hydroxide

nCSF-1, natural macrophage colony stimulating factor

ng, nanogram

NH₄OH, ammonium hydroxide

NHS, normal human serum

NIH, National Institutes of Health

nm, nanometer
NT, not tested
OD, optical density
PAGE, polyacrylamide gel electrophoresis
PBS, phosphate buffered saline
PFU, plaque forming unit
PGE, prostaglandin E
PMUE, pregnant mouse uterine extract
r, recombinant
RNA, ribonucleic acid
SA, synergistic activity
SDS, sodium dodecyl sulfate
SEM, standard error of the mean
SRBC, sheep red blood cell
TFA, trifluoroacetic acid
TNF, tumor necrosis factor
Tris-HCl, tris (hydromethyl) aminomethane hydrochloride
U, units
UV, ultraviolet
VSV, vesicular stomatitis virus

INTRODUCTION

The differentiation of cells of the monocyte-macrophage lineage from bone marrow progenitors to mature effector populations in peripheral tissues is an extremely complex developmental process. Subpopulations of progenitor cells, which differ in their capacities for replication (self-renewal) and differentiation, must respond to a variety of distinct growth factors to divide and/or differentiate. Once released into the circulation, monocytes can enter the peripheral tissues and mature into macrophages under the influence of environmental signals. These mature macrophages also exhibit a heterogeneous spectrum of morphological and functional phenotypes characteristically associated with successive levels of macrophage differentiation. The purpose of this introduction is to present our current understanding of macrophage differentiation. First, a discussion of macrophage progenitor subpopulations will be presented. This will be followed by a description of the growth factors which have been shown to participate in the development of cells committed to the monocyte-macrophage lineage. Lastly, an overview of mature macrophage heterogeneity as a reflection of a differentiation hierarchy will be addressed.

Heterogeneity of hematopoietic progenitors

Hematopoiesis, the complex process of blood cell formation, requires specific conditions for the generation of eight distinct mature cell lineages: erythrocytes, megakaryocytes, T cells, B cells, eosinophils, basophils, granulocytes, and monocyte-macrophages. The generation of these lineages requires stimulation of a heterogeneous population of progenitors by a variety of growth factors with hormone-like effects (i.e., active at 10^{-10} M concentrations), as well as a microenvironment, generated by bone marrow stromal cells, conducive to hematopoiesis (Dexter et al., 1985). Early work by Till and McCulloch (1961) demonstrated the existence of a very primitive stem cell which, following injection into

irradiated recipients, was capable of colony formation in the spleen. This stem cell was called "Colony-Forming Unit-spleen" (CFU-s). Examination of the splenic nodules which formed revealed that these stem cells were capable of giving rise to cells of the megakaryocyte, erythrocyte, granulocyte, and macrophage lineages, and thus, represents a multi-potent progenitor cell. Although these CFU-s stem cells were capable of multi-lineage differentiation, they also exhibited the capacity to replicate without differentiation and were, therefore, capable of reconstituting irradiated mice (Dexter *et al.*, 1985). Subpopulations of CFU-s were subsequently characterized on the basis of size and density (Till and McCulloch, 1961; Haskill *et al.*, 1970) and were shown to differ with respect to their capacity to form splenic nodules at varying times following injection (Till and McCulloch, 1961; Dexter *et al.*, 1985). Thus, some CFU-s could give rise to nodules only within 8 days of injection, whereas others induced nodules as late as 12 days following injection into irradiated recipients (Till and McCulloch, 1961; Dexter *et al.*, 1985). This heterogeneity was interpreted as representing a progression from a stem cell with extensive replicative capacity to one which was more restricted in its replicative ability (Wolf and Priestly, 1986). For this reason, these subpopulations of CFU-s cells were referred to as "early" CFU-s and "late" CFU-s stem cells.

The development of *in vitro* culture conditions which employed a soft agar assay and conditioned medium from a variety of tissues sources (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966) led to the identification of progenitors which were generically referred to "Colony-Forming Unit-culture" (CFU-c). These progenitors differed from those described *in vivo* (CFU-s) in that they exhibited increased lineage restriction and a more limited capacity for self-renewal (Quesenberry and Levitt, 1979). Thus, the CFU-c was hypothesized to result from the expansion and differentiation of the early multi-potent stem cells, CFU-s (Rosendaal *et al.*, 1979; Metcalf and Moore, 1973). In other words, the proliferation and differentiation of early stem cells (CFU-s) is believed to result in progeny (CFU-c) which are less multi-potent (i.e., they possess a more limited ability to reconstitute irradiated animals; Metcalf and Moore, 1973). The establishment of the *in vitro* colony assay allowed for further examination of CFU-c

progenitors and provided a means by which additional progenitors and progenitor subpopulations could be distinguished. Subpopulations of progenitors have also been described within the CFU-c progenitor pool based on differences in size or density (Worton *et al.*, 1969; Haskill *et al.*, 1970; Bol and Williams, 1980), as well as the development of lineage-restricted progeny in response to Colony Stimulating Factors (CSFs; Metcalf and MacDonald, 1975).

One such CFU-c progenitor subpopulation which exhibits increased lineage restriction is the "Granulocyte-Macrophage Colony-Forming Cell" (GM-CFC). Upon stimulation, this population of progenitors gives rise to mature granulocytes or macrophages. This finding established a close link between these two mature cell lineages. The survival and proliferative expansion of this population of progenitors is completely dependent upon the presence of specific growth and differentiative factors, the CSFs. The use of the soft agar assay and micromanipulation of daughter cells during early colony formation demonstrated the bipotent nature of the GM-CFC (Metcalf and Burgess, 1982). In addition, experiments have shown that following 3 - 5 mitoses in the presence of a lineage-specific CSF, i.e., granulocyte-macrophage CSF (GM-CSF), granulocyte CSF (G-CSF), or macrophage CSF (CSF-1), irreversible commitment to that lineage occurs (Metcalf and Burgess, 1982). Even within this progenitor population, however, heterogeneity has been observed based on differences in size, density, and CSF sensitivity of various progenitors (Williams and van den Engh, 1975; Metcalf and MacDonald, 1975; Bol *et al.*, 1979; Bol and Williams, 1980; Bertoncello *et al.*, 1981). In addition to the bipotent progenitor described by Metcalf and Burgess (1982), these investigators also demonstrated the existence of more mature progenitors with more severe lineage restriction. These progenitors were responsive to a single lineage-specific CSF, CSF-1 or G-CSF and were called "Macrophage Colony-Forming Cells" (M-CFC) and "Granulocyte Colony-Forming Cells" (G-CFC), respectively.

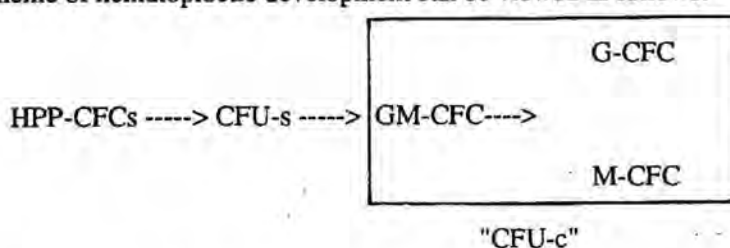
While, the original utilization of the soft agar colony assay allowed for the discrimination of multiple progenitor populations at different stages of maturity, subsequent modifications (see below) allowed for the demonstration of another early, primitive stem cell

which was referred to as a "High Proliferative Potential Colony-Forming Cell" (HPP-CFC). Although individual species of CSF have been described as giving rise to lineage-specific populations (reviewed in Williams and Jackson, 1977; McNiece *et al.*, 1986), it is clear that these factors can act synergistically with other cell-derived factors which may result in the development of very large colonies with high proliferative potential, HPP-CFCs (Bradley and Hodgson, 1979; Baines *et al.*, 1981; Kriegler *et al.*, 1982; Bartelmez *et al.*, 1985; Bartelmez and Stanley, 1985; Bertoncello *et al.*, 1986; Koike *et al.*, 1986; McNiece *et al.*, 1986; Stanley *et al.*, 1986; Beck *et al.*, 1987; Bertoncello *et al.*, 1987; Mochizuki *et al.*, 1987; Pluznik *et al.*, 1987; Zucali *et al.*, 1987). The original demonstration of these primitive progenitors by Bradley and Hodgson (1979) utilized crude CSF-1-containing extracts from pregnant mouse uterine tissue and human spleen cell conditioned medium (HSCM) as the source of the factor which provided the synergistic activity (Bradley and Hodgson, 1979). HSCM alone was unable to induce colony formation *in vitro*. Subsequently, HPP-CFC were shown to possess the capacity to reconstitute irradiated recipients (Hodgson *et al.*, 1982; McNiece *et al.*, 1987).

The methods used to demonstrate the existence of HPP-CFC progenitors differ markedly from study to study: colony formation in soft agar (Bol *et al.*, 1979; Bradley and Hodgson, 1979; Bertoncello *et al.*, 1986; McNiece *et al.*, 1986; Bertoncello *et al.*, 1987), inclusion of a variety of sera in the culture medium for greater nutritional support (Bol *et al.*, 1979; Bradley and Hodgson, 1979; Baines *et al.*, 1981; Madonna and Vogel, 1985; Hagan *et al.*, 1985; McNiece *et al.*, 1986; Schwartz *et al.*, 1986), radiolabeled cytokine-receptor interaction for the demonstration of newly expressed CSF receptors following stimulation with a synergistic factor (Bartelmez *et al.*, 1985; Bartelmez *et al.*, 1985b; Stanley *et al.*, 1986), as well as the demonstration of high proliferative responses of bone marrow progenitors as measured by thymidine incorporation (Mochizuki *et al.*, 1987). Within the soft agar assay alone, a spectrum of methodologies has been utilized to study the expansion of HPP-CFC progenitors (Bradley and Hodgson, 1979; Baines *et al.*, 1981; Madonna and Vogel, 1985; Hagan *et al.*, 1985; McNiece *et al.*, 1986; Schwartz *et al.*, 1986). Most of these require an enrichment for HPP-CFC by pre-treatment of mice with the

cytotoxic drug 5-FU, as well as inclusion of high concentrations of normal human serum (in addition to fetal calf and horse serum) in the culture medium. As has been noted for other progenitor populations, there appear to be subpopulations of HPP-CFCs as well. HPP-CFC subpopulations have been characterized on the basis of their specific factor requirements (Bradley and Hodgson, 1979; McNiece *et al.*, 1982; Bradley *et al.*, 1985; Koike *et al.*, 1986; McNiece *et al.*, 1987), differential sensitivity to 5-FU (Bradley *et al.*, 1985), their ability to be enriched within the 5-FU-derived population of bone marrow cells (Bradley *et al.*, 1985), and their requirements for auxillary serum factors for *in vitro* expansion (L.A. Falk and S.N. Vogel, submitted). Based on the work of McNiece *et al.* (1987), it is believed that HPP-CFCs give rise to CFU-s.

This brief summary of hematopoiesis illustrates that the heterogeneity within the bone marrow progenitor compartment is based on a hierarchical scheme of reduced self-renewal capacity accompanied by increased lineage restriction. Within each hierarchical level, subpopulations of progenitors have been demonstrated. To a large degree, the ability to demonstrate this subpopulational complexity is limited by our methods of detection and the ability to supply the stimulatory requirements necessary for expansion of a particular progenitor population *in vitro*. Based on the hematopoietic scheme described thus far, a proposed scheme of hematopoietic development can be viewed as follows:



Since this project focuses on the mechanisms by which macrophage heterogeneity develops, the remainder of this introduction will examine hematopoiesis and maturation as it pertains solely to macrophage development and differentiation.

Heterogeneity as reflected by multiple species of CSF

The development of soft agar culture conditions which support the clonal proliferation of granulocyte and/or macrophage populations, led to the recognition that such cells were incapable of autonomous proliferation and required stimulation by specific regulatory molecules (reviewed by Metcalf, 1987). Therefore, these molecules were collectively referred to as "granulocyte-macrophage" CSFs. There are four CSFs which have been shown to stimulate progenitors to develop into mature granulocytes or macrophages: Multi-CSF [Interleukin 3 (IL 3); Ihle *et al.*, 1983], granulocyte-macrophage CSF (GM-CSF; Metcalf, 1980), granulocyte CSF (G-CSF; Metcalf and Nicola, 1983), and macrophage CSF (CSF-1; Stanley and Heard, 1977). Of these four CSF species, three have been shown to give rise to mature macrophages: IL 3 (to a limited extent; Ihle *et al.*, 1983), GM-CSF (Metcalf, 1980), and CSF-1 (Stanley and Heard, 1977). These three CSFs share some common features: (i) each CSF is a glycoprotein, although the sugar moiety is not required for biological activity or receptor binding (Das and Stanley, 1982; Ihle *et al.*, 1982; Burgess *et al.*, 1985; Metcalf, 1985); (ii) all three CSFs contain disulfide bonds which are necessary for biological activity (Stanley and Heard, 1977; Das *et al.*, 1980; Burgess *et al.*, 1985; Gough *et al.*, 1985); (iii) the biological activities of these molecules are in the range of 10^{-10} to 10^{-13} M (Stanley and Guilbert, 1981; Ihle *et al.*, 1982; Gough *et al.*, 1985); and (iv) the GM-CSF and IL 3 genes are located on Chromosome 11 in the mouse and, therefore, the possibility of coordinate regulation (with respect to the transcription, synthesis, or action of these two CSFs) has been proposed (Gough *et al.*, 1984; Ihle and Silver, 1985). Although the location of the gene which encodes the murine CSF-1 is not known, studies of the human CSF-1 gene have placed it on Chromosome 5 along with the genes for GM-CSF, IL 3, and the CSF-1 receptor (Le Beau *et al.*, 1986; Pettenati *et al.*, 1987).

Interleukin 3 (IL 3) is produced primarily by mitogen-stimulated T cells and has been detected in conditioned medium from the myelomonocytic cell line WEHI-3B (Ihle *et al.*, 1982). In addition, IL 3 has been cloned (Fung *et al.*, 1984), has a molecular weight of 23 - 28,000

daltons, and has been shown to bind to a receptor of molecular weight 60 - 75,000 daltons. The IL 3 receptor is present in low numbers on bone marrow cells, granulocytes, monocytes, and eosinophils (Palaszynski and Ihle, 1984; Metcalf, 1986). In addition to its ability to stimulate granulocyte or macrophage colony formation, IL 3 has also been shown to stimulate CFU-s replication, as well as act upon multi-potent cells to form colonies which contain erythrocytes, eosinophils, megakaryocytes, and mast cells (Hapel *et al.*, 1985; Kindler *et al.*, 1985).

GM-CSF has also been shown to give rise to granulocytes, macrophages, or mixed granulocyte-macrophage colonies. GM-CSF has been detected and/or purified from a number of tissue sources, i.e., endothelial cells (reviewed by Metcalf, 1987), T cells (Ruscetti and Chervenick, 1975; Prystowski *et al.*, 1983; Gough *et al.*, 1985), macrophages (Thorens *et al.*, 1987) and the EL-4 thymoma (DeLamarter, 1985). GM-CSF has been cloned from T cells (Gough *et al.*, 1984), has a molecular weight of 23,000 daltons, and binds to high and low affinity GM-CSF-specific receptors (Walker and Burgess, 1985). GM-CSF receptors have been found on all granulocytes, macrophages, and eosinophils (Walker and Burgess, 1985; Metcalf, 1986). Molecular cloning of GM-CSF has shown that the gene which encodes GM-CSF contains two initiation sites, and, therefore, has the potential to produce two different mRNA transcripts (Stanley *et al.*, 1985). The mRNA for the longer transcript contains two translation initiation codons and hydrophobicity analysis of the amino acid sequences of the two possible translation products is consistent with the potential for the protein product of the longer transcript to be membrane-bound (Gough, 1985; Stanley *et al.*, 1985). This finding is of great interest given the evidence to suggest that in the bone marrow stroma, local concentrations of CSF in the microenvironment and cell contact may be involved in the control of granulopoiesis (Allen and Dexter, 1976; Zipori, 1981). In addition, the choice of promoter usage may reflect preferential transcription of either the "long" or "short" mRNA species. In this regard, the promoter which gives rise to the shorter transcript appears to be used preferentially following Concanavalin A stimulation of T cells (Gough *et al.*, 1985) and may reflect a molecular mechanism for increased production of secreted GM-CSF versus

membrane-bound forms of GM-CSF.

The third CSF which has been shown to stimulate macrophage production is the lineage-restricted CSF, macrophage CSF (CSF-1). CSF-1 has been shown to be a product of fibroblasts, embryonic yolk sac, whole embryo, and pregnant mouse uterine extract (reviewed by Metcalf, 1986). CSF-1 has been purified to homogeneity from murine L929 fibroblast conditioned medium (Stanley and Heard, 1977; Waheed and Shadduck, 1979), has a molecular weight of between 40 - 70,000 daltons due to extensive glycosylation (Stanley and Heard, 1977; Metcalf, 1986), and binds to a receptor with a molecular weight of 165,000 daltons (Morgan and Stanley, 1984). The gene for the CSF-1 receptor has also been shown to be identical to the cellular proto-oncogene *c-fms* (Sherr et al., 1985), and is expressed in large numbers on cells of the monocyte-macrophage lineage (Guilbert and Stanley, 1980; Byrne et al., 1981; Stanley et al., 1983; Shadduck et al., 1983). Unlike the previously described CSFs, CSF-1 is a homodimer of protein subunits, each with an unglycosylated molecular weight of 14,500 daltons (Stanley and Guilbert, 1981). Only the dimeric form has been shown to be biologically active (Stanley and Guilbert, 1981). In contrast to the species specificity exhibited by human GM-CSF (Metcalf, 1985), human CSF-1 has been shown to be fully active on murine bone marrow cells (Stanley et al., 1975). Recombinant murine CSF-1 is not available at this time, but many investigators have recently taken advantage of the species cross-reactivity of CSF-1 and have utilized recombinant human CSF-1 (Kawasaki et al., 1985; Ladner et al., 1987) in their studies on murine progenitors. As is the case for murine GM-CSF, the cDNA for human CSF-1 indicates the possible existence of multiple transcriptional species; the protein product of the larger transcript contains sequences, that upon translation, are characteristic of a transmembrane portion (Kawasaki et al., 1985; Ralph et al., 1986). Thus, as indicated for GM-CSF, CSF-1 may also exist in a membrane-bound form as well as a secreted form.

Although the CSFs have many general structural similarities, they share no sequence homology (Gough et al., 1984). While most of the granulocyte-macrophage progenitors are bipotent and can respond to more than one CSF, they exhibit considerable heterogeneity with

respect to: (i) the number of progeny each generates; and (ii) the concentration of CSF required to stimulate cell division (reviewed by Metcalf, 1985). This latter finding is consistent with the differences in receptor number on individual progenitors observed by autoradiography (Walker and Burgess, 1985). Although these factors have been shown to act on similar populations of progenitors, this apparent redundancy may indicate a subtle and complex control system by which progenitor cell proliferation and subsequent differentiation is regulated. In addition, this may provide a mechanism for competitive and/or potentiating interactions between different species of CSFs. Thus, the observed heterogeneity within the progenitor populations observed at each hierarchical step, coupled with the apparent cytokine redundancy observed within the granulocyte/macrophage CSF family, may provide a fine control mechanism for the production and activation of selected subsets of mature progeny.

Functional and differentiative heterogeneity in mature macrophage populations

The role of macrophages in an animal's innate defense against bacterial invasion has been described by a number of investigators for Brucella (Pomales-Lebron and Stineberg, 1957; Elber *et al.*, 1957; Holland and Pickett, 1958), Salmonella (Hobson, 1957; Howard, 1961; Sato *et al.*, 1961), and for Listeria (Mackaness, 1962; Armstrong and Sword, 1964). In all cases, macrophages from immune animals displayed a greatly enhanced capacity for microbicidal activity *in vitro*, in contrast to normal macrophages which were quickly destroyed by unrestricted intracellular bacterial growth. In addition, *in vivo* administration of potent "macrophage activating agents", such as Bacillus Calmette Guerin (BCG) or *in vitro* treatment of peritoneal macrophages with crude lymphokine preparations, induced macrophages to become microbicidal or tumoricidal (Hibbs, 1976; Nogueira *et al.*, 1978). Thus, injection of a variety of stimuli, which induce acute or chronic inflammation in the peritoneal cavity of animals, resulted in the *in situ* accumulation of macrophages which were morphologically, biochemically, and functionally different from resident peritoneal macrophages (Karnovsky

et al., 1975; Cohn, 1978). Thus, highly microbicidal macrophages were considered to be "fully activated" and represented the culmination of the differentiation process (Cohn, 1978). These findings established the importance of the "activated" macrophage as effector cells responsible for killing of intracellular parasites and tumor cells. The role of the macrophage in host defense has, therefore, prompted much work to determine the mechanisms by which macrophages become fully activated and the acquisition of characteristics along this differentiative pathway.

Heterogeneity within mature macrophage populations has been hypothesized to be a result of macrophages at various differentiation states which culminate in the fully mature or "activated" macrophage (Cohn, 1978). Most of the early work which led to an association of specific functional or topographical markers with different stages along the activation pathway involved examination of heterogeneous populations of macrophages present in the peritoneal cavity of untreated mice versus mice which had received various stimulants or eliciting agents intraperitoneally. Morphologically, macrophages exhibited an increase in cell size and increased membrane ruffling following activation (Cohn, 1978). In addition, work by a number of investigators (Lazdin *et al.*, 1975; Beelen *et al.*, 1978; Bursucker and Goldman, 1982) demonstrated that resident peritoneal macrophages exhibited different enzymatic activities, i.e., peroxidase and 5'-nucleotidase, than peritoneal exudate macrophages. In these studies, resident macrophages were shown to exhibit a high degree of 5'-nucleotidase activity and peroxidase activity which was localized to the nuclear envelope, in contrast to low 5'-nucleotidase activity and peroxidase activity which was localized to the cytoplasmic granule in exudate macrophages. These findings provided the first evidence that biochemical differences might be utilized as "markers" for the differentiation state of a macrophage.

The initial characterization of macrophages as "mononuclear phagocytes" by Van Furth and Cohn (1968) implied that the capacity to ingest foreign particles was a functional hallmark of this cell type. Phagocytosis may occur by two different mechanisms: non-receptor-mediated uptake of particles or receptor-mediated ingestion (reviewed by Rosenstreich, 1981).

Macrophages have been shown to possess two major classes of receptors for the Fc portion of the immunoglobulin molecule, one specific for the binding of the Fc portion of IgG_{2a} and the other for the binding of IgG_{2b} (Unkeless, 1977). These Fc receptors play an important role in those macrophage functions which require participation of antibodies, such as antibody-dependent cellular cytotoxicity and phagocytosis of opsonized particles (Unkeless, 1977). The observation that peritoneal macrophages elicited by various agents differed with respect to their quantitative expression of Fc receptors, led to the hypothesis that Fc receptor expression might also serve as a marker for macrophage differentiation (Bianco *et al.*, 1975). These investigators showed that resident peritoneal macrophages had very low Fc receptor capacity when compared to macrophages elicited by a variety of "stimulating agents," such as thioglycollate. In 1980, Neuman and Sorg demonstrated, *in vitro*, that one of the first functional properties which macrophages acquire during their development from bone marrow progenitors under the influence of crude CSF preparations is that of phagocytosis. Based on the kinetics of appearance, the earliest phagocytic capacity acquired during macrophage differentiation is that of non-specific, non-receptor-mediated phagocytosis. With increasing differentiation, macrophages develop the additional, more specific capacity of phagocytosis via Fc receptors (Neuman and Sorg, 1980). Since these studies were carried out, extensive evidence has accumulated in the literature to indicate that induction of Fc receptor expression is highly regulated (reviewed by Friedman and Vogel, 1983). Fc receptor expression can be augmented by addition of exogenous IFN- α/β or IFN- γ and results in an increased capacity of macrophages to phagocytose opsonized particles (reviewed by Friedman and Vogel, 1983). In addition, Fc receptor expression can be modulated by IFN- α/β produced endogenously during macrophage differentiation from bone marrow progenitors in the presence of CSF-1 (Moore *et al.*, 1984; Warren and Vogel, 1985a). Thus, increased phagocytic capabilities, and specifically, an increase in the capacity to bind and phagocytose via Fc receptors, is now considered to be another marker of macrophage differentiation.

In addition to Fc receptors, macrophages also possess a second family of receptors

which can recognize and bind products of the third component of the complement cascade (Bianco *et al.*, 1975). The receptors for C3b and its degradation products are distinct from the Fc receptors (Bianco *et al.*, 1975; Ross *et al.*, 1983). Although Fc-mediated phagocytosis was shown to be increased in inflammatory macrophages (Bianco *et al.*, 1975), an even more profound increase was observed in the ability of macrophages to bind and ingest C3b coated-particles (Bianco *et al.*, 1975; Cohn, 1978). While complement-coated erythrocytes bind equally well to resident and inflammatory macrophages, ingestion of the C3b-coated erythrocytes was only observed in more "activated" macrophages (Bianco *et al.*, 1975; Morland and Kaplan, 1977). Therefore, the authors proposed that in activated macrophages, but not in less differentiated macrophages, the C3b receptor may be associated with contractile elements in the cytoplasm. Alternatively, it was proposed that the density of C3b receptors on the less differentiated macrophages was adequate for binding of complement-coated particles, but below the levels required for ingestion (Bianco *et al.*, 1975). For these reasons, the ability to bind and phagocytose via C3b receptors was defined as a more advanced marker of macrophage differentiation than Fc receptor expression.

Another cell surface marker of macrophages which has become associated with more highly differentiated macrophages is the ability to express products of the Major Histocompatibility Complex (MHC) called Class II molecules or Ia antigens. Ia antigens are integral membrane proteins which are involved in the recognition reactions that permit the immune response system to distinguish self from non-self (reviewed by Steinmetz and Hood, 1983). Individual Class II MHC molecules (or Ia antigens) are composed of two non-covalently associated, polypeptide chains, denoted α and β . Each of these subunits has two external domains, a transmembrane domain, and a smaller cytoplasmic domain (reviewed by Steinmetz and Hood, 1983). Ia molecules are normally expressed on a limited number of cell types, i.e., primarily B cells and macrophages, and exhibit extensive serological polymorphism (reviewed by Steinmetz and Hood, 1983). The expression of Ia antigens is essential for the appropriate presentation of antigen by antigen-presenting cells, such as macrophages, to antigen-specific

T cells (Rosenthal and Shevach, 1973; Heber-Katz *et al.*, 1982; Beller, 1984; Unanue and Allen, 1987). Although the expression of Ia antigens is critical for a macrophage to serve as an antigen-presenting cell, the expression of Ia is not sufficient for driving antigen-specific T cell proliferation. Prior to the "presentation" of antigen, in the context of the Ia molecule, the macrophage must ingest and "process" the foreign antigen. The ability of a macrophage to process antigen requires lysosomal degradation, a biochemical "processing" event that changes the structure of the antigens so that they will be recognized by the immune system in a form distinct from that of the native protein (Unanue and Allen, 1987). The immunogenic peptides which are generated are then exported to the cell surface for presentation in the context of an Ia molecule (Germain, 1981; Weinberger *et al.*, 1981; Unanue *et al.*, 1984). Recent evidence demonstrates a requirement for additional secreted proteins and cell surface markers for stimulation of optimal T cell proliferation, such as, secreted and/or membrane-bound Interleukin 1 (IL 1; Mizel and Ben-Zvi, 1980; Kurt-Jones *et al.*, 1985, Virgin *et al.*, 1985; Gerrard *et al.*, 1987; Humme, 1987)

As was found for the expression of Fc and C3b receptors, macrophages elicited by potent activating agents, such as infection with Listeria monocytogenes, were found to express high levels of cell surface Ia (Beller *et al.*, 1980). In addition, soluble mediators found in lymphokine-rich supernatants were also found to induce Ia-positive macrophage populations *in vivo* (Scher *et al.*, 1980). In subsequent studies, resident, proteose peptone-elicited, and thioglycollate-elicited peritoneal macrophage populations were shown to express Ia following stimulation of macrophage cultures by crude lymphokine preparations *in vitro* (Beller, 1984). However, their findings indicated that the culture period required for induction of Ia positive macrophages was longer for resident than for elicited macrophages. This led Beller and his colleagues to propose that the observed difference in the kinetics of Ia antigen induction reflected the degree of differentiation of the macrophage population prior to lymphokine treatment and was confirmed by measuring 5'-nucleotidase activity in the different macrophage populations examined (Beller, 1984).

Much attention has focused on the identity of the soluble mediator(s) which induce Ia antigen expression. Using natural, and then subsequently cloned reagents, IFN- γ has been shown to be a principal lymphokine responsible for upregulating Ia antigen expression on macrophages (Steinman *et al.*, 1980; Steeg *et al.*, 1980; Birmingham *et al.*, 1982; King and Jones, 1983; Vogel *et al.*, 1983; Warren and Vogel, 1985a), as well as on other cell types (Koch *et al.*, 1984; Berrih, *et al.*, 1985; Groenewegen *et al.*, 1986; Ruff *et al.*, 1986). Recently, another lymphokine, Interleukin 4 (IL 4), was also shown to increase Ia antigen expression on macrophages (Crawford, *et al.*, 1987). The enhanced expression of Ia antigen has also been correlated with increased Ia-dependent accessory functions by a number of investigators (Steeg *et al.*, 1980; Tzchoval *et al.*, 1981; Birmingham *et al.*, 1982; Beller, 1984). In addition to the augmentation of Ia expression induced by IFN- γ , Ia antigen expression has also been shown to be under negative regulation. Two substances which can down-regulate induced Ia antigen expression are IFN- α/β (Ling *et al.*, 1985; Inaba *et al.*, 1986; Fertsch *et al.*, 1987) and prostaglandins (Snyder *et al.*, 1982; Tripp *et al.*, 1986). Both of these soluble factors have been shown to antagonize the effects of IFN- γ -induced Ia antigen expression *in vitro*. Thus, augmentation of Ia antigen expression on cells which normally express low levels of Ia by potent inflammatory stimuli, has become accepted as a "higher order" marker of macrophage differentiation.

An additional characteristic associated with "highly activated" macrophages is that of acquisition of tumoricidal activity. Hibbs *et al.* (1977) provided the first evidence that the activation of macrophages to a fully tumoricidal state required a precise series of signals to the differentiating macrophage. Briefly, Hibbs and his colleagues proposed that induction of maximal tumoricidal activity was a multi-signal event: macrophages were first "primed" into a stimulated, but non-cytolytic state by lymphokine(s), which were generically referred to as "MAF". Once "primed", the macrophage could be activated to full tumoricidal capacity by a "trigger" signal. Environmental stimuli, such as Gram negative lipopolysaccharide (LPS), were identified as potent "trigger" signals. Neither the priming signal alone nor the trigger

signal alone resulted in macrophage activation to kill tumor cells. In addition, the priming signal had to be provided prior to or simultaneously with the trigger signal to induce tumoricidal activity (Ruco and Meltzer, 1978; Hogan and Vogel, 1988).

Based on work carried out by Weinberg *et al.* (1978), it was demonstrated that priming signals differed in their potency: as the potency of the priming signal is increased, one could achieve activation with lesser concentrations of trigger signal. For instance, when macrophages were elicited with the potent activating agents BCG or *Corynebacterium parvum*, as little as 0.5 - 1 ng/ml of LPS was required to trigger full tumoricidal activity. However, if a less potent eliciting agent (such as proteose peptone) were administered, approximately 1000-times more LPS was required to induce full tumoricidal activity. The concentration of trigger signal required to activate proteose peptone-elicited macrophages could be significantly lowered by first treating the cells with preparations of "MAF". Following *in vitro* stimulation by MAF, proteose peptone-elicited macrophages required only 1 - 10 ng/ml of LPS to trigger full tumoricidal activity. Thus, differentiation of macrophages to a fully tumoricidal state depends on the correct sequence of priming and triggering signals as well as complementary levels of the two signals. These findings have since been confirmed and extended in many laboratories (Weinberg *et al.*, 1978; Pace and Russell, 1981; Pace *et al.*, 1983a; Schreiber *et al.*, 1983; Pace *et al.*, 1983b; Hogan and Vogel, 1987).

Using the P815 mastocytoma as a target tumor cell, Pace *et al.* (1983; 1983b) and Schreiber *et al.* (1983) defined IFN- γ as the principal form of Hibb's proposed MAF in lymphokine and T cell hybridoma supernatants. With the availability of recombinant IFN- γ (rIFN- γ), these observations were defined more precisely: maximal tumoricidal activity was induced in proteose peptone-elicited macrophages (derived from LPS-responsive C3H/HeN mice) with 1 - 10 U/ml rIFN- γ and as little as 0.3 ng/ml LPS. Neither rIFN- γ alone nor LPS alone induced any significant increase in tumoricidal activity in these macrophages. If macrophages were derived from the LPS-hyporesponsive C3H/HeJ strain, protein-free LPS could not be used as a "trigger" signal; however, alternate second signals, such as heat-killed

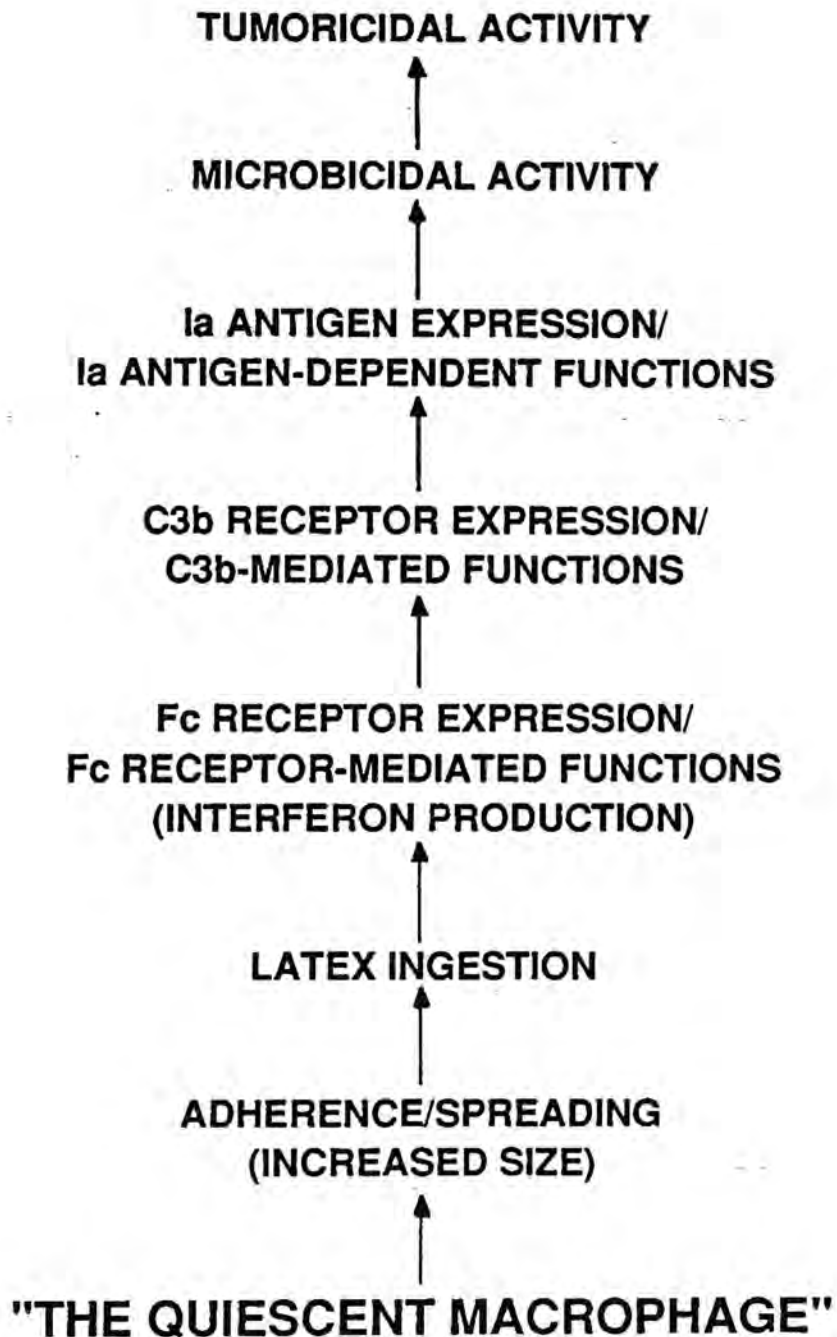
L. monocytogenes (Pace *et al.*, 1985a), protein-rich, butanol-extracted LPS (Pace *et al.*, 1983b; Hogan and Vogel, 1987), or purified lipid A-associated protein (Hogan and Vogel, 1987), were fully efficacious as "trigger" signals. The mechanism(s) by which macrophages mediate tumor killing is unclear; however, a number of soluble factors produced by highly activated macrophages, such as superoxide anion and hydrogen peroxide (Murray and Cartelli, 1983; Murray *et al.*, 1983; Nathan *et al.*, 1983), arginase (Currie, 1978), and tumor necrosis factor (Jadus *et al.*, 1986; Decker *et al.*, 1987), have been implicated in macrophage tumoricidal and/or microbicidal activity. The demonstration that IFN- γ alone is an insufficient stimulus for inducing tumoricidal activity, coupled with the finding that not all Ia-positive macrophage populations are capable of killing tumor cells *in vitro* (Blumenthal *et al.*, 1983), have led to the conclusion that tumoricidal activity is a more highly differentiated function than the capacity to express Ia antigen. Taken collectively, these findings have placed specific macrophage markers/functions along a differentiative hierarchy (Figure 1).

The previous sections have focused on the different levels of heterogeneity which are associated with macrophage differentiation: precursor heterogeneity, heterogeneity among Colony Stimulating Factors, and the biochemical, functional, and cell marker heterogeneity observed within mature macrophage populations. The existence of mature macrophage populations which differ markedly in their functional abilities and organ distribution has prompted much speculation and debate as to the nature, location, and generation of these subpopulations of cells. We hypothesized that the action of two distinct species of CSF on progenitor cells might give rise to populations of macrophages which differ functionally. Therefore, the goal of this dissertation was to focus on the development of macrophage heterogeneity through the use of bone marrow-derived progenitors stimulated with highly purified or recombinant preparations of CSF-1 or GM-CSF. Through a side by side comparison, we have examined the outcome of stimulating bone marrow progenitors with these two distinct species of CSF. The results will be sub-divided into two major sections: the effects of GM-CSF

versus CSF-1 on progenitor cell responsiveness and the morphological and functional characterization of the resultant macrophage populations.

Figure 1. Hierarchical scheme of macrophage differentiation. Figure 1 illustrates a hierarchical scheme of macrophage differentiation based on that originally published by Cohn (1978) and modified to include subsequent work by Neumann and Sorg (1980) and Beller (1984).

THE "FULLY ACTIVATED" MACROPHAGE



MATERIALS AND METHODS

REAGENTS

COLONY STIMULATING FACTORS (CSFs)

GM-CSF preparations. A natural preparation of murine GM-CSF (nGM-CSF) was purchased from Genzyme (specific activity: 2.2×10^6 U/mg; Boston, MA). This material was "functionally pure" (free of IL 2, IL 3, and IFN- γ activities) and was used in some of the initial studies prior to the availability of murine recombinant GM-CSF (rGM-CSF). Preparations of murine rGM-CSF were the kind gifts of Biogen, SA (Geneva, Switzerland) and Immunex, Corp. (Seattle, WA) and had specific activities of 1.2×10^7 U/mg and 5×10^7 U/mg respectively. Human recombinant GM-CSF (hGM-CSF) was provided courtesy of Immunex, Corp. (Seattle, WA) and had a specific activity of 4×10^7 U/mg.

CSF-1 preparations. Natural murine CSF-1 was purified from serum-free culture supernatants of murine L929 fibroblasts by a modification of the procedure originally described by Stanley and Heard (1977). Murine L929 fibroblasts were grown to confluency in the presence of Eagle's Minimum Essential Medium (EMEM; M.A. Bioproducts, Walkersville, MD) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT), 2 mM glutamine (GIBCO Laboratories, Grand Island, NY), 15 mM HEPES (Research Organics, Inc., Cleveland, OH), 0.02% sodium bicarbonate (Fisher Scientific, Fairlawn, NJ), penicillin and streptomycin (P/S; 100 IU/ml and 100 μ g/ml respectively, GIBCO Laboratories), and 50 μ g/ml gentamicin (Quality Biological Inc., Gaithersburg, MD) at 37°C in 5% CO₂. All tissue culture reagents were purchased as "endotoxin free" lots (≤ 0.01 ng/ml). When the fibroblasts reached confluency, serum-containing medium was removed and the cells were washed three times with Earle's Balanced Salt Solution (EBSS; GIBCO Laboratories). The monolayers were then re-cultured in serum-free EMEM for an additional 7 days. The replacement of serum-containing medium with serum-free medium prior to column purification was

estimated to result in an intrinsic purification of approximately 1000-fold. Following this period of serum deprivation, culture supernatants were harvested and purified by an additional four-step purification procedure, to be described below, which included hydroxylapatite chromatography, diethylaminoethyl (DEAE) Sephacel chromatography, Affi-Gel 202 chromatography, and high performance liquid chromatography (HPLC).

Fractions from each column purification step were screened for biological activity using a bone marrow proliferation assay described elsewhere (Moore and Rouse, 1983). For this assay, C3H/HeJ (Jackson Laboratories, Bar Harbor, ME) bone marrow cells were obtained by flushing the tibias and femurs of mice with serum-free medium. The cells were centrifuged at 684 X g at room temperature for 10 minutes. Cells were resuspended in serum-free medium and 5 ml of cell suspension was layered onto 5 ml of Lymphocyte Separation Medium (LSM; Litton Bionetics, Charleston, SC) in a 15 cc conical tube and centrifuged at 554 X g, at room temperature, for 20 minutes. Following centrifugation, mononuclear cells were harvested from the interface and washed in serum-free medium. The LSM-purified cells were resuspended in RPMI (M.A. Bioproducts) supplemented with 10% FCS, 2 mM glutamine, 30 mM HEPES, 0.4% sodium bicarbonate, penicillin and streptomycin (100 IU/ml and 100 µg/ml respectively) and were plated into 96-well culture plates (Falcon, Oxnard, CA) at a cell density of 1×10^4 cells/well (i.e., 0.1 ml of a 1×10^5 cells/ml). Bone marrow cells were incubated in the presence of serial two-fold dilutions of column fractions (0.1 ml) for three days, pulsed with ^3H -thymidine (0.5 µCi/well; specific activity= 6.7 Ci/mol; NEN, Boston, MA) and harvested 5 hours later onto glass-fiber filter strips (Cell Harvester; Brandel, Gaithersburg, MD). This procedure allowed for the assessment of relative CSF-1 activity of individual column fractions based on thymidine incorporation by bone marrow cells as determined by liquid scintillation counting.

For each batch of CSF-1 purified, approximately three liters of serum-free supernatants were concentrated approximately 10-fold by Pellicon concentration

(Millipore Corp., Milford, MA) using a membrane which had as its lower retention limit a molecular weight of 10,000 daltons. The concentrate was subjected to hydroxylapatite column chromatography (Bio-HT; BioRad, Richmond, CA). The concentrate was applied directly to a 15 ml column which contained Bio-HT matrix previously equilibrated with approximately 5 column volumes of serum-free EMEM using a duostaltic pump at a flow rate of 25 ml/hour (Haake Buchler Instruments, Inc., Saddle Brook, NJ). CSF-1-containing material was eluted using a 150 ml linear gradient which ranged from serum-free EMEM to 150 mM sodium phosphate buffer (pH 7.2) with a flow rate of 25 ml/hour. Twenty minute fractions were collected (LKB 2111 Multitrac fraction collector; LKB, Rockville, MD) and the absorbance (OD_{280}) of each fraction detected using a spectrophotometer (LKB 8300 Uvicord II, LKB). Active fractions eluted early in the gradient from the Bio-HT column (up to 33 mM phosphate). In the original method of Stanley and Heard (1977), CSF-1 activity was separated by a batch-elution technique using hydroxylapatite. The smaller starting volume of material generated as a result of Pellicon concentration allowed us to use a linear gradient for the elution of CSF-1 activity. The use of a linear gradient resulted in sharper separation of CSF-1 activity from contaminating proteins since the CSF-1 activity could be more selectively eluted by a more well-defined salt gradient.

The active fractions from the Bio-HT column were dialyzed against 10 mM phosphate-10 mM NaCl buffer (pH 7.2) prior to DEAE chromatography (Pharmacia, Uppsala, Sweden). CSF-1 activity was eluted from a 15 ml DEAE column using a two-step salt gradient. The DEAE column was pre-equilibrated with 10 mM phosphate-10 mM NaCl buffer. Following sample application, the DEAE column was first washed with 75 ml of 10 mM phosphate-60 mM NaCl followed by 75 ml of 10 mM phosphate-0.3 M NaCl. The CSF-1 activity was completely contained within the 10 mM phosphate-0.3 M NaCl portion of the step gradient. The two-step gradient proved to be as efficient as the linear gradient described by Stanley and Heard (1977) in the removal of CSF-1 from the DEAE column.

The active fractions recovered following DEAE chromatography were dialyzed

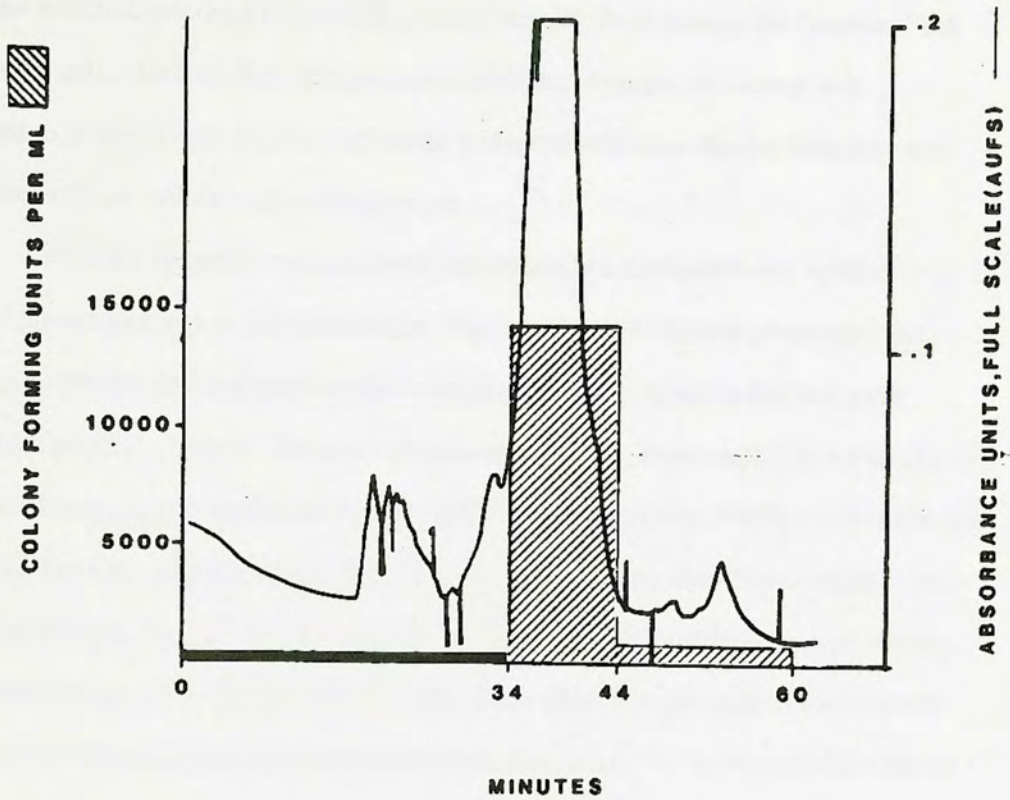
against 0.05 M acetate buffer (pH 5.0) and applied to a 15 ml Affi-Gel 202 column (BioRad, Richmond, VA) which had been pre-equilibrated with 0.05 M acetate buffer. The starting material from the DEAE column was loaded onto the Affi-Gel 202 column and was washed with 75-100 ml 0.05 M acetate buffer to ensure complete recovery of CSF-1. The material which passed through the column, as well as the material contained in the wash, was pooled and concentrated (Amicon, Danvers, MA) to reduce the volume of material prior to its application to HPLC. The addition of the Affi-Gel 202 column to our purification scheme allowed for the retention of IFN- α/β while allowing the CSF-1 activity to pass through the column (Stewart, 1981). In the earlier work by Stanley and Heard (1977), it had not been appreciated that L929 fibroblasts produce IFN- α/β constitutively at very low levels. For this reason, we modified the original Stanley and Heard procedure to include the Affi-Gel 202 column specifically to insure removal of this important immunoregulatory cytokine.

The concentrated CSF-1-containing material obtained from the Affi-Gel 202 column was next dialyzed for 4 hours into 0.1% trifluoroacetic acid (TFA; Sigma, St. Louis, MO), pH 2.0 in preparation for reverse phase HPLC. This material was applied to a C-18 reverse-phase HPLC column (Waters Corp, Milford, MA) using a Model 6000A solvent delivery system and Model U6K injector (Waters Corp.). The gradient conditions were controlled using a Data Module and Systems Controller (Waters Corp.). The CSF-1 activity was eluted from a 30-minute, 0-50% acetonitrile/0.1% TFA gradient with a flow rate of 0.5 ml/minute. CSF-1 activity eluted as a single peak of protein between 34 and 44 minutes as detected using a variable wavelength UV detector (Waters Corp.). All reagents were HPLC grade.

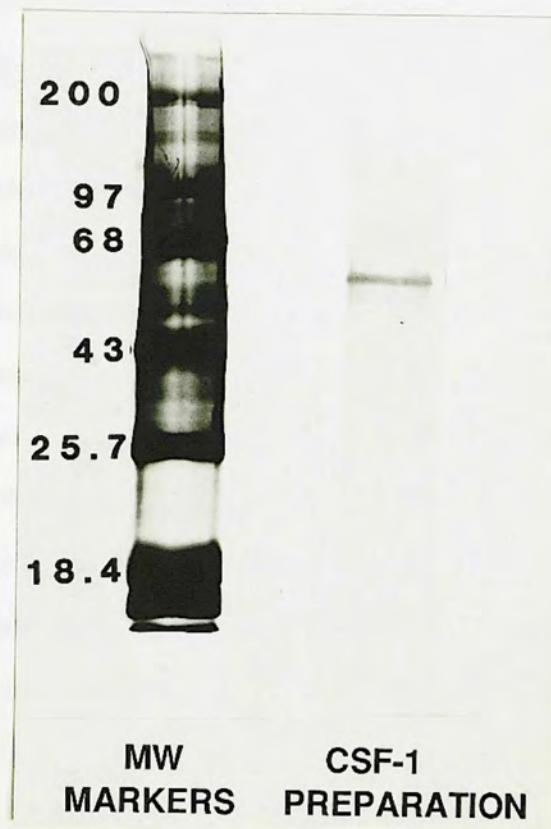
The elution profile of CSF-1 following the HPLC step of the five-stage purification scheme is shown in Figure 2A. This material was analyzed further using non-reducing polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining of the gel (Figure 2B). For the latter, preparations of CSF-1 taken from the active HPLC fractions were electrophoresed through a 10% resolving gel in the presence of sodium dodecyl

Figure 2. Elution profile and gel electrophoresis of murine CSF-1 following HPLC chromatography. CSF-1-containing material was purified further by C-18 reverse-phase HPLC chromatography and the protein elution profile following a 0.5 ml/minute, 0-50% acetonitrile/0.1% TFA gradient is shown (A). The CSF-1-activity associated with each fraction was determined by colony formation in soft agar and is also shown in panel A. The HPLC-purified CSF-1 was electrophoresed (10% SDS-PAGE), the proteins developed by silver stain and a photograph of the stained gel (with the corresponding molecular weight markers) is shown in panel B.

A.



B.



sulfate (SDS) according to the method of Laemmli (1970). CSF-1 preparations in sample buffer [0.5M Tris-HCl (pH 6.8), glycerol, 10% SDS, and 0.05% bromphenol blue] were added to the gel and electrophoresed (Protein II gel apparatus; BioRad) through the "stacking" gel (2.5 ma per gel). Once the dye front had entered the resolving gel, the current was increased to 30 ma per gel and electrophoresis was terminated once the dye front migrated approximately one inch from the end of the gel.

Following electrophoresis, gels were transferred to a methanol/water solution (1:1, v/v) and placed on a rocker platform (Bellco, Vineland, NJ) overnight in preparation for silver stain. For detection of proteins by silver stain, gels were rinsed in distilled water prior to the addition of silver. Silver solution was prepared by dissolving 0.8 g AgNO_3 in 4.0 ml distilled water, and once dissolved, 1.4 ml of NH_4OH and 21.0 ml 0.36% NaOH were added dropwise. Once the silver was dissolved, 75 ml of distilled water was added making a total volume of 100 ml. The gel was then placed in the silver solution and rocked for 20 minutes. At the end of this period, the silver solution was removed and the gel washed with distilled water for 15 minutes. This wash step was repeated three times. To develop the gel, 0.25 ml of formaldehyde (37%) was added to 2.5 ml 1% citric acid and brought to a final volume of 500 ml with distilled water. The gel was allowed to develop on a rocker platform and once developed sufficiently (approximately 15 - 20 minutes), the developer removed and methanol (1:1, v/v) added to the gel to stop the reaction.

Our five-step purification scheme, therefore, resulted in the generation of a highly purified preparation in which the major protein peak following HPLC co-eluted with CSF-1 activity, and the apparent molecular weight of the predominant CSF species was shown to be approximately 58,000 daltons. This molecular weight is consistent with those reported by others (Stanley and Heard, 1977; Burgess and *et al.*, 1985; and Das and Stanley, 1982). This material was also tested and found to be free of antiviral activity. Thus, the only other immunoregulatory cytokine known to be present in supernatants from L929 cells, IFN- α/β , was removed by this procedure.

In some studies, a less purified, natural, CSF-1 preparation was utilized to allow for increased replications of experiments. The preparation of this material has been described elsewhere (Warren and Vogel, 1985a). Briefly, murine L929 supernatants which contained 10% FCS were subjected to a 40% ammonium sulfate precipitation. The precipitate was sedimented by centrifugation (16,000 X g, 30 minutes), and the resulting supernatant was dialyzed against 0.05 M acetate buffer prior to Affi-Gel 202 column chromatography.

Human recombinant CSF-1 (HUrCSF-1; specific activity: $> 5 \times 10^7$ U/mg), a kind gift of Cetus Corp. (Emeryville, CA), was used to confirm the results obtained using the highly purified, murine nCSF-1.

Colony assay for the quantitative determination of activity in various CSF preparations. The activity of the various CSF-1 and GM-CSF preparations (both natural and recombinant) was determined by the method of Stanley *et al.* (1972). One $\times 10^5$ LSM-purified bone marrow cells were cultured in a soft agar system which consisted of RPMI-10% FCS and 0.35% Bacto-Agar (Difco Laboratories, Detroit, MI). Serial dilutions of CSF preparations (0.2 ml) were added to 6-well tissue culture plates (Costar, Cambridge, MA) and were overlaid with 1.0 ml agar-medium mixture which contained 1×10^5 cells/ml. Colonies (≥ 25 cells) were scored following a 7-day incubation period at 37°C and 5% CO_2 . The activity of each preparations was based on the number of colony-forming units/ml (CFU/ml) and was determined by correcting the number of colonies counted along the linear portion of the dilution curve for the dilution factor of the CSF in that well.

INTERFERONS

Interferon (IFN)- α/β (specific activity $\geq 5 \times 10^8$ U/mg) was generously provided by Dr. M. Pauker (Medical College of Pennsylvania, Philadelphia, PA). In some studies, serial two-fold dilutions of anti-murine IFN- α/β antibody (NIH Reference Reagent No.

G-024-501-568) or a control antibody (NIH Reference Reagent No. G-025-501-568) were added to macrophage monolayers in a final volume of 0.2 ml. Recombinant Interferon- γ (rIFN- γ ; specific activity $> 1.3 \times 10^7$ U/mg) was kindly provided by Genentech, Inc. (South San Francisco, CA). All interferon activities were confirmed using a standard antiviral assay (Vogel *et al.*, 1982) and the activity determined following comparison of the sample's activity to that of the NIH Murine Interferon Standard (Reference Reagent No. G002-904-511).

ANALYSIS OF MACROPHAGE PROGENITOR CELLS

Quantitation of GM-CSF- and CSF-1-responsive progenitor cells. Bone marrow progenitor cells from C3H/HeJ (Lps^d) and C3H/OuJ (Lps^H) mice (Jackson Laboratories) were purified on LSM as described above. The number of CSF-responsive progenitor cells was determined using a double-agar overlay, semisolid colony assay originally described by MacVittie and McCarthy (1977), and modified subsequently by Madonna and Vogel (1985). The bottom layer consisted of a source of CSF (0.25 ml) contained in 60 mm gridded culture dishes (Nunc; PGC Scientific, Gaithersburg, MD). The CSF was overlaid with 2.0 ml of 0.5% agar-medium mixture which contained the following: 15% CMRL 1066 (10X concentrate; GIBCO Laboratories), 2.0% sodium pyruvate (100X concentrate; M.A. Bioproducts), 0.006% L-asparagine (GIBCO Laboratories), 0.0042% L-serine (GIBCO Laboratories), 0.44% sodium bicarbonate (Mallinckrodt, Inc., St. Louis, MO), 0.6% tryptic soy broth (Difco Laboratories), 1% antibiotic-antimycotic solution (100X concentrate; GIBCO Laboratories), 20% heat inactivated FCS, 10% heat inactivated horse serum (HyClone), and 0.5% Bacto-Agar (Difco Laboratories). The upper layer contained 5×10^4 LSM-purified bone marrow cells in 2.0 ml of 0.33% agar-medium mixture. In this standard assay system for the enumeration of bone marrow progenitors, cultures were incubated at 37°C in 6% CO₂ for 10 days and colonies (≥ 50 cells) were counted using an inverted microscope (40X

magnification).

To quantitate the number of GM-CSF- and CSF-1-responsive progenitors in the spleen, the same general methodology as described above for the enumeration of bone marrow progenitors was utilized. Spleens from C3H/HeJ and C3H/OuJ mice were dissociated into single cell suspensions in separate petri-dishes which contained serum-free RPMI. The cell suspensions were aspirated repeatedly, using a pasteur pipet, to disperse cell aggregates. The single cell suspensions were centrifuged at 684 X g at room temperature for 10 minutes, resuspended in 5.0 ml in serum-free RPMI and layered onto a 5.0 ml LSM gradient. The LSM gradients were centrifuged (554 X g, room temperature, 20 minutes) and the mononuclear cells were harvested from the interface. Previous reports have shown that the number of macrophage progenitors in the spleen is significantly lower than the number found in the bone marrow (MacVittie and Weinberg, 1980) and therefore, the number of input spleen cells per culture was increased 10-fold compared to that used for culturing of bone marrow cells. Thus, 5×10^5 LSM-purified spleen cells were added to each culture dish in the identical manner as that described above for the detection of CSF-responsive bone marrow progenitors. Cultures were incubated for 10 days at 37°C in 6% CO₂ and colonies (> 50 cells) were counted using an inverted microscope (40X magnification).

Quantitative determination of responsive progenitors following CSF deprivation.

The number of progenitors retaining proliferative capacity following 1-7 days of CSF deprivation was examined using a modification of the double agar-overlay system described above for detection of GM-CFC and M-CFC. Briefly, cultures contained a bottom layer of medium or CSF in 0.5% agar-medium (D0). These cultures were then overlaid with 5×10^4 bone marrow cells in 0.33% agar-medium and incubated for 10 days in 37°C and 6% CO₂. Cultures which received medium on D0 were overlaid with either rGM-CSF or nCSF-1 in 0.33% agar-medium mixture at D1, D2, D3, D4, and D7 of culture. All cultures were

examined for colony formation 10 days from the initial seeding of bone marrow cells (D0), as well as 10 days from the addition of CSF to the individual cultures.

Stimulation and detection of bone marrow progenitors with high proliferative potential (HPP-CFC). In some experiments, bone marrow cells were examined for the stimulation of HPP-CFC using the cell culture method described above for the detection of GM-CFC and M-CFC progenitors. For these experiments, cultures were incubated at 37° C in 6% CO₂ for 10 days (the standard incubation time for the development of GM-CFC- or M-CFC-derived colonies) and 12 days for the quantitation of colonies derived from HPP-CFCs. Colony size was determined at 40X magnification using an eyepiece micrometer and colony diameter was scored as < 2 mm or ≥ 2 mm. It should be noted that unlike culture conditions reported previously to support the proliferative expansion of HPP-CFCs (Bradley and Hodgson, 1979), there is no human serum present under our standard culture conditions. In these experiments, cytokine preparations were used at 1000 units (U) per culture. This concentration of CSF-1 and GM-CSF were determined in studies described herein to be in excess for the generation of GM-CFC- and M-CFC-derived colonies of < 2 mm diameter (Figure 3). Since recent reports by Mochizuki *et al.* (1987) have shown that GM-CSF can synergize with Interleukin 1 (IL 1) for the proliferative expansion of HPP-CFCs, we included this cytokine combination as a positive control in our assay system for the development of HPP-CFC-derived colonies (≥ 2 mm). Human recombinant IL 1α (rIL 1) was generously provided by Hoffmann LaRoche, Inc. (specific activity: 1.5 x 10⁶ U/mg; Nutley, NJ). The concentration of rIL 1 used in this study was also 1000 U/culture which was shown in preliminary studies to be on the dose plateau for induction of HPP-CFC colony formation when used in combination with rGM-CSF. In certain experiments, bone marrow cells from mice treated with 150 mg/kg of the cytotoxic drug, 5-fluorouracil (5-FU; Sigma) were used at a cell concentration of 5 x 10⁴ viable, LSM-purified bone marrow cells per culture dish.

In some experiments, pooled normal human serum (NHS) was included in the standard culture medium at a final concentration of 25%. NHS was obtained from normal, healthy, type B donors (kindly provided by Dr. Susan Langreth, Uniformed Services University of the Health Sciences, Bethesda, MD). Additional experiments also included human spleen cell conditioned medium (HSCM) at a final concentration of 10%. HSCM was prepared by the method of Metcalf and Johnson (1981) and was generously provided by Dr. Thomas J. MacVittie (Armed Forces Radiobiology Research Institute, Bethesda, MD). This concentration of HSCM had been found in Dr. MacVittie's laboratory to synergize maximally with CSF-1 for the formation of HPP-CFC-derived colonies.

Signal separation for stimulation of HPP-CFC. Cultures were established as described above with either rGM-CSF or CSF-1 on Day 0 and were then overlayed on subsequent days with 1.0 ml of 0.33% agar-medium which contained the alternate species of CSF. Cultures were examined 12 days from the initiation of cultures and 12 days post addition of the second CSF. Colonies were scored as < 2 mm or ≥ 2 mm using an eyepiece micrometer.

Culture conditions and morphological analysis of macrophages derived from bone marrow progenitors under the influence of CSF in liquid culture. Bone marrow progenitors were also cultured in liquid culture using a modification of the method of Warren and Vogel (1985a). LSM-purified bone marrow cells were obtained from C3H/HeJ or C3H/OuJ mice (The Jackson Laboratories), as described above, and were cultured at 1×10^7 cells per flask (for CSF-1) or 3×10^7 cells per flask (for GM-CSF) in 25-cm² flasks (Corning Glass Works, Corning, NY) in the presence of 10 ml of EMEM-10% FCS supplemented with 250 U/ml of CSF (Day 0). Three times as many bone marrow cells had to be seeded in GM-CSF cultures to obtain yields of mature macrophages equivalent to those seen in CSF-1-derived cultures seeded at 1×10^7 cells/flask. These data will be presented in the Results section. After 24 hours (Day 1), nonadherent cells from each 25-cm² flask were

transferred to a 75-cm² flask (Corning Glass Works, Corning, NY) and supplemented with an additional 10 ml of medium which contained CSF (250 U/ml). The cultures were again supplemented on Day 4 with an additional 10 ml of CSF-containing medium. After a total of 7 days (D7) in culture, macrophages were removed enzymatically with the neutral protease, Dispase II (Boehringer Mannheim, Indianapolis, IN) and gentle scraping with a rubber policeman. The resulting cell suspensions were examined morphologically by visual inspection of modified Wright-stained (Diff-Quik; American Scientific Products, McGaw Park, IL), cytocentrifuge-prepared slides (Cytospin 2; Shandon, Sewickly, PA). The cells which resulted from culture in either GM-CSF or CSF-1 were also examined for cell size distribution at various times over the 7 day culture period. Cell sizing profiles were obtained using a Coulter Counter Model ZM and Coulter Channelyzer 100 equipped with a X-Y Recorder 4 (Coulter Electronics, Inc., Hialeah, FL), which was calibrated according to the manufacture's specifications.

FUNCTIONAL ASSAYS FOR THE ANALYSIS OF GM-CSF- AND CSF-1-DERIVED MACROPHAGES

To study the functional and differentiative characteristics of CSF-1- and GM-CSF-derived macrophages, bone marrow progenitors were grown in liquid culture in the presence of either cytokine for 7 days as described above. The adherent (D7) macrophages were removed enzymatically and re-plated at known densities in the absence of exogenous CSF for further examination. In experiments where unequal numbers of replicates are indicated, the lesser number of replicates is reflective of experiments in which both GM-CSF- and CSF-1-derived macrophages were examined simultaneously. The additional experiments used to generate the pooled data were unmatched data points.

Assay for phagocytosis of latex beads by macrophages. Macrophages derived from bone marrow cultures under the influence of GM-CSF or CSF-1 were examined for their capacity to ingest latex beads as described elsewhere (Vogel and Rosenstreich, 1979). Two $\times 10^5$ mature (Day 7) macrophages were resuspended in RPMI-2% FCS and re-cultured in 8-well Lab-Tek tissue culture chambers (Miles Laboratories, Inc., Naperville, IL) for 4 hours. Following a 4-hour incubation to allow for adherence of macrophages, the culture supernatants were removed and the monolayers were incubated for 1 hour at 37° C with 0.4 ml per chamber of latex beads (Dow Chemical Company, Indianapolis, IN; 1.1- μ diameter, diluted 1:500 in medium). Cells were washed vigorously five times with medium to remove non-internalized-beads. The cultures were methanol-fixed, Wright-stained (Diff-Quik), and the number of ingested particles per macrophage scored under oil immersion (1000X magnification).

Assays for Fc-receptor-mediated phagocytosis and binding of opsonized erythrocytes by macrophages. Fc-mediated phagocytosis was quantified visually by a modification of the method of Vogel and Rosenstreich (1979). Two $\times 10^5$ macrophages were cultured in 8-well Lab-Tek tissue culture chambers and treated with medium or IFN- α/β for 48 hours. Opsonized SRBC were prepared by washing SRBC with saline three times, and 1×10^9 washed SRBC were incubated with 10 μ l of rabbit anti-sheep erythrocyte antibody (purified IgG; Cordis Labs Corp., Miami, FL) for 1 hour in a 37° C water bath. Following this incubation, opsonized SRBC were washed free of unbound antibody and were resuspended to a volume of 25 ml with medium. Macrophage monolayers were incubated with 0.2 ml of opsonized SRBC for 1 hour at 37° C, at which time, non-internalized SRBC were lysed with 0.2 ml of an ammonium chloride lysing solution (8.29 g/l ammonium chloride, 1.0 g/l potassium bicarbonate, and 0.0375 g/l ethylenediaminetetraacetic acid; EDTA). Cultures were washed once with medium and then fixed, stained, and scored under oil immersion (1000X magnification) for the number of SRBC ingested per cell.

Analysis of Fc-mediated binding of IgG-coated SRBC was carried out as described above for the analysis of Fc-mediated phagocytosis. SRBC were washed, opsonized, resuspended to 25 ml, and 1.5×10^{-3} M iodoacetic acid (IAA; Sigma) was added to the SRBC suspension just prior to the addition of opsonized SRBC to macrophage cultures. The presence of IAA has been shown to block Fc-mediated phagocytosis without inhibiting the binding of opsonized erythrocytes (Walker and Demus, 1975). Following an 1 hour incubation period of macrophage monolayers and opsonized SRBC, cultures were washed to remove unbound SRBC, fixed, stained, and scored (1000X magnification) for the number of macrophages which bound opsonized SRBC, as well as the number bound per cell.

Sensitivity of GM-CSF- and CSF-1-derived macrophages to vesicular stomatitis virus (VSV). Two $\times 10^5$ mature (Day 7) macrophages were re-cultured in 96-well plates and allowed to adhere for 6 hours as described previously (Vogel *et al.* 1986). At this time, supernatants were gently aspirated and 0.1 ml of a VSV suspension [Indiana strain; Multiplicity of Infection (M.O.I.) of 0.1 or 1.0], diluted in medium, was added to each well. Macrophage cultures were infected for 24 hours, at which time, the supernatants were removed and frozen at -70° C for subsequent analysis of virus yield. The adherent cells were fixed for 10 minutes with 5% formaldehyde and stained for 10 minutes with 0.05% crystal violet to demonstrate cytopathic effect (CPE).

Culture supernatants from virus-infected macrophages were examined for viral replication as described by Vogel and Fertsch (1987). Briefly, L929 fibroblasts were grown to confluency in 6-well culture plates (Falcon). Cells were infected for 1 hour at room temperature with 1.0 ml of virus sample diluted in EMEM-10% FCS. After the 1-hour adsorption period, the contents of each well were removed and the monolayer overlaid with 1.0 ml of phenol red-free EMEM which was supplemented with sodium bicarbonate, glutamine, penicillin and streptomycin, 5% FCS and 1% Noble agar (Difco Laboratories). Once solidified, the cultures were incubated at 32° C for 2 days in 5% CO_2 . Plaques were

developed by overlaying each culture with 1 ml phenol red-free EMEM supplemented as described above, but with the addition of neutral red dye (GIBCO Laboratories; 1:150 dilution). Cultures were incubated at 37° C for 4 to 6 hours and then scored for viral plaque formation. Each supernatant was assayed at multiple dilutions in duplicate.

To detect intracellular VSV in infected cells, rGM-CSF- or CSF-1-derived macrophages were re-plated at 2×10^5 per well in 8-well Lab-Tek tissue culture chambers and were allowed to adhere for 4 - 6 hours. Following this adherence stage, culture supernatants were removed and 0.1 ml of VSV suspension, diluted in medium (M.O.I. of 0.1), was added to each well. Cultures were infected for 6 hours, acetone fixed for 10 minutes, and the slides frozen at -20° C. Prior to fluorescent staining, culture slides were re-hydrated with 0.1 ml of a solution which contained phosphate buffered saline (PBS), 1% bovine serum albumin (BSA; Sigma), and 5% goat serum (GS; GIBCO Laboratories) for 15 minutes at room temperature. Cultures were washed with PBS/BSA/GS and incubated with rabbit anti-VSV antibody (Lee Biomolecular Research Inc., San Diego, CA) diluted 1:50 in PBS/BSA/GS for 30 minutes at room temperature. At a final concentration of 1:10,000 in a total volume of 0.1 ml, this preparation of anti-VSV antibody was found to inhibit CPE in L929 fibroblasts infected with VSV (M.O.I. = 0.1). Prior to addition of a fluorescent secondary antibody, cultures were subjected to three 15 minute washes with PBS/BSA/GS. Cultures were then incubated with 0.05 ml of rhodamine-conjugated, goat anti-rabbit IgG antibody (Cooper Biomedical Inc., Malvern, PA) diluted 1:50 in PBS/BSA/GS for 30 minutes at 37° C in the dark. Cultures were washed for two 15 minute periods and one 30 minute period using PBS/BSA/GS (in the dark). Cultures were examined for specific fluorescence using an Olympus BH-2 epi-fluorescent microscope (40X magnification). Control cultures consisted of: (i) uninfected macrophage cultures treated with the anti-VSV antibody and rhodamine-conjugated secondary antibody and (ii) VSV-infected cultures treated with rhodamine-conjugated secondary antibody only.

Quantitative determination of interferon activity in macrophage culture

supernatants. The detection of interferon in macrophage culture supernatants was performed as described elsewhere (Vogel *et al.*, 1982). Briefly, serial two-fold dilutions of supernatant samples were prepared in 0.05 ml volumes in flat-bottomed 96-well culture plates (Falcon). To each well was added 0.05 ml of L929 fibroblasts (1×10^5 cells per well). After a 24 hour incubation period at 37°C and 5% CO_2 , the supernatants were aspirated and the cells infected with 0.1 ml of VSV in EMEM-5% FCS at an M.O.I. of 0.1. At 24 hours post-infection, the cells were washed with EBSS and fixed for 10 minutes with 5% formaldehyde. The cells were stained for 10 minutes with 0.05% crystal violet, and the plates subsequently washed with tap water. Three controls were included in each assay: (i) uninfected, medium-treated L929 cells (cell control), (ii) infected, medium-treated L929 cells (virus control), and (iii) a titration of 100 U/ml NIH murine fibroblast IFN- α/β (Reference Reagent No. G-002-904-511). Reciprocal titers (expressed as U/ml) were based on a comparison of the sample's endpoint with the titration of the NIH standard.

Measurement of Ia antigen expression. Ia antigen expression was measured by three methods: direct complement-mediated cytotoxicity (Warren and Vogel, 1985a), an ELISA (enzyme-linked immunosorbent assay; Warren and Vogel, 1985a) and by fluorescent antibody analysis through the use of the ACAS 470 (Wade *et al.*, 1986; described below). Direct complement-mediated cytotoxicity provides a measure of the percentage of Ia-positive macrophages. The ELISA assay provides a relative measure of total Ia antigen expression per culture without regard to the number of Ia-positive cells. Fluorescent antibody analysis provided by the anchored cell analysis and sorting station (ACAS 470) provides a direct measure of Ia antigen density per cell. As for all of the other functional assays described thus far, rGM-CSF- or CSF-1-derived macrophages were removed from flasks enzymatically on Day 7 and re-cultured in the absence of colony stimulating factor

at 2×10^5 cells/well (for the direct cytotoxicity and ELISA assays) or at 1×10^5 cells/well (for fluorescent antibody analysis). Cultures were treated with medium only or medium plus rIFN- γ (5.0 U/ml) and were examined at 24, 48, and 72 hours following treatment. Comparability of cell densities of medium-treated and IFN- γ -treated cultures was verified by examination of total cell protein, as determined by the method of Lowry (1951).

In the cytotoxicity assay (Warren and Vogel, 1985a), the cells were first treated with a monoclonal anti-Ia^k antibody [10-2.16 hybridoma (Oi *et al.*, 1978); American Type Culture Collection, Rockville, MD; affinity-purified as described elsewhere (Fultz *et al.*, 1982)] and incubated for 45 minutes at 4° C. Subsequently, the antibody was aspirated from the cells and rabbit complement (Low-Tox; Cedarlane Laboratories, Ontario, Canada; diluted 1:12 in RPMI-2% FCS) was added to the monolayer. Following a 45 minute incubation at 37° C, the complement was aspirated and trypan blue was added. The number of Ia-positive cells was determined by counting the number of trypan blue-positive cells. The percentage of Ia-positive cells was determined following subtraction of the percentage of trypan blue-positive cells observed in cultures treated with complement alone. Complement control cultures typically contained 3 - 4% trypan blue-positive cells and also provided a measure of the viability of the cells following one to three days in culture. In addition, cultures were also examined following treatment with an irrelevant monoclonal antibody (anti-Ia^d) plus complement. These cultures consistently exhibited $\leq 5\%$ trypan blue-positive cells.

For the ELISA assay (Warren and Vogel, 1985), macrophage monolayers were fixed with 1% paraformaldehyde in PBS for 20 minutes at room temperature. Following fixation, cells were washed extensively with EBSS-1% FCS and were then incubated with the same preparation of monoclonal anti-Ia^k antibody or an irrelevant monoclonal (anti-Ia^d) antibody for 45 minutes at 4° C. Cells were then washed, and incubated with a peroxidase-conjugated secondary antibody (goat anti-mouse IgG, F(ab')₂; Cooper Biomedical, Inc., Malvern, PA) for 45 minutes at room temperature, followed by washing.

The amount of enzyme-conjugated antibody that remained associated with the cells was measured by the addition of the substrate orthophenylenediamine and the incubation of cultures in the dark for 30 minutes at room temperature. The enzymatic reaction was stopped with the addition of 8 N sulfuric acid and the absorbance read at 490 nm (Bio-Tek EIA Reader, Burlington, VT). The absorbance (OD_{490}) measured in cultures treated with an irrelevant primary antibody (anti-Ia^d) was < 0.09 absorbance units.

Ia density per cell was visualized by fluorescent antibody analysis through the use of the ACAS 470 (Wade *et al.*, 1986; Meridian Instruments Inc., Okemos, MI). Briefly, macrophage monolayers were treated as described above for Ia detection by ELISA except that in lieu of a peroxidase-conjugated secondary antibody, cultures were incubated with a fluorescein-conjugated secondary antibody (goat anti-mouse IgG, F(ab')₂; Cooper Biomedical Inc, Malvern, PA) for 45 minutes at 4^o C in the dark. The following ACAS 470 parameters were utilized: wavelength = 488 nm, dichroic filter = 510 nm, step size = 2.0 μ , laser power = 200 milliwatts, and scan strength = 10%. Background fluorescence was determined by treatment of macrophage monolayers with fluorescein-conjugated secondary antibody only.

Detection and quantitation of Ia-specific cytoplasmic RNA. Ia-specific mRNA was isolated and examined according to the procedure of Fertsch *et al.* (1987). Briefly, 8×10^6 bone marrow-derived macrophages were removed enzymatically after 7 days in culture with rGM-CSF or nCSF-1. The cells were washed three times with ice-cold EBSS and the cell pellet resuspended in an ice-cold isotonic solution. The cells were then lysed and the nuclei cleared from the lysate by centrifugation. The resulting cytoplasmic supernatant was subjected to a series of phenol and chloroform: phenol (1:1; v/v) extractions to isolate cytoplasmic RNA. The RNA was ethanol-precipitated overnight and the amount of RNA isolated was quantitated by measurement of absorbance at 260 nm. Equal loading of gel lanes with RNA was also verified by ethidium bromide staining of gels for Northern blots.

Following ethanol precipitation, RNA preparations were examined for Ia-specific mRNA by Northern blot and by slot blot analysis. For Northern blot analysis, RNA was denatured, electrophoresed on a 1% agarose-formaldehyde gel, and transferred to nitrocellulose. For slot blot analysis, RNA was denatured and applied to nitrocellulose filters with a Minifold Slot Manifold (Schleicher and Schueller, Keene, NH). Nitrocellulose filters were baked at 80° C for 2 hours and then prehybridized overnight at 42° C. Filters were hybridized with 2×10^6 cpm/ml of ^{32}P -CTP-labelled probe (A_{α}^d ; described in detail in Fertsch *et al.*, 1987; Specific activity = $\sim 1 \times 10^9$ cpm/ μg) at 42° C for 18 - 24 hours. Following hybridization, blots were washed extensively, and were exposed to Kodak-XAR film at -70° C with intensifying screens. A densitometer (Hoefer Scientific Instruments, San Francisco, CA) was used to scan the autoradiograms and the peak areas from the recorded scans were calculated using a digitizer (Hewlett-Packard, Co., Fort Collin, CO) as described elsewhere (Fertsch *et al.*, 1987). ORA cells (a constitutively Ia-positive, macrophage cell line) were the kind gift of Dr. Carol L. Reinisch (Tufts University School of Veterinary Medicine, Boston, MA). These cells served as a source of constitutive Ia message which was included as a positive control for Ia mRNA detection.

Measurement of antigen-induced T cell proliferation by GM-CSF- and CSF-1-derived macrophages. The ability of antigen-pulsed rGM-CSF- and nCSF-1-derived macrophages to induce antigen-specific T cell proliferation was examined by a modification of the procedure of Lee and Wong (1980). Recombinant GM-CSF- and nCSF-1-derived macrophages were harvested at Day 7 and were re-cultured at either 1×10^5 or 2×10^5 cells/well in 96-well plates in the absence of CSF. Cultures were treated with medium only or medium plus rIFN- γ for 48 - 72 hours. Following treatment, monolayers were pulsed with 200 $\mu\text{g}/\text{ml}$ of filter-sterilized keyhole limpet hemocyanin (KLH; Calbiochem-Behring, La Jolla, CA) for 4 hours at 37° C. The macrophage cultures were irradiated at 1500 rads (Gammacell 40, $^{137}\text{Cesium}$ irradiation unit) at the end of the antigen-pulsing period.

Following irradiation, the monolayers were washed and co-cultured with 2×10^5 nylon wool-purified lymph node cells (Weinblatt *et al.*, 1981) isolated from C3H/HeJ mice which had been primed 6 - 7 days previously with 50 μ g/foot pad of KLH in Complete Freund's Adjuvant (Difco Laboratories). The proportion of macrophages to T cells used in this study was based on the approximate range found by others to give strong, antigen-specific, T cell proliferation using nylon-wool purified T lymphocytes as the responder population (Lee and Wong, 1980; Beller, 1984). Macrophages and lymph node cells were co-cultured for 4 days and were then pulsed with ^3H -thymidine for 20 hours (0.5 μCi /well; NEN, Boston, MA). Cells were harvested onto glass fiber filters and the radioactivity determined by liquid scintillation counting. To analyze the data statistically, a two-way analysis of variance (ANOVA) was carried out and the differences between treatment groups were compared using a test for least significant differences (LSD; Snedecor and Cochran, 1967). Differences between treatment groups were taken to be statistically significant if $p \leq 0.05$.

Prostaglandin E (PGE) production and assay. Following culture of rGM-CSF- and nCSF-1-derived macrophage cultures with medium alone, supernatants were harvested at various times and were assayed for production of PGE by a radioimmunoassay which has been described in detail elsewhere (Wahl, 1980). Briefly, dilutions of culture supernatants were incubated in the presence of ^3H -PGE₂ (12,000-15,000 cpm; Amersham, Arlington Heights, IL with a specific activity of 120 Ci/mmol). Rabbit anti-PGE₂ (Miles Laboratory, Elkhart, IN) was then added and the tubes incubated for 1 - 2 hours at 37°C. Following this incubation, goat anti-rabbit serum (Meloy Laboratory, Springfield, VA) was added to the tubes and incubated for 18 hours at 4°C. After incubation, the tubes were centrifuged at 1900 X g for 30 - 45 minutes at 4°C. The precipitate was solubilized in 50 mM Tris (pH 9.0) and the radioactivity measured by liquid scintillation counting. The percentage of binding

was calculated as follows:

$$\text{Percentage Bound} = \frac{\text{standard curve cpm} - \text{background cpm}}{\text{total bound cpm} - \text{background cpm}}$$

The percentage cpm bound for each sample was then compared to the percentage cpm bound in the standard curve and the amount of PGE present determined by extrapolation from the standard curve.

Assay for macrophage-mediated tumoricidal activity. Killing of ^{51}Cr -labeled P815 mastocytoma cells was measured by using a 16 hour ^{51}Cr -release assay which has been described previously (Hogan and Vogel, 1987). Briefly, P815 tumor target cells (the kind gift of Dr. J. Pace, Kansas University Medical Center, Kansas City, KS) were labeled for 1 - 3 hours at 37°C with $500\ \mu\text{Ci}$ of ^{51}Cr per 5×10^6 cells (^{51}Cr -labeled, sodium chromate; ICN Biomedicals, Inc., Irvine, CA; specific activity = $500\ \text{mCi/mg}$), washed once by centrifugation, and allowed to "leak" for 1 hour at 37°C in RPMI-10% FCS. Following a 24 hour incubation period of macrophage cultures in the presence of a "priming" signal (rIFN- γ) and/or a "trigger" signal (butanol-extracted lipopolysaccharide; But-LPS), ^{51}Cr -labeled tumor targets (1×10^4) were added. But-LPS was extracted by the method of Morrison and Leive (1973) and has been shown to serve as a "trigger" signal for both LPS-responder (Lps^{R}) and non-responder (Lps^{d}) macrophages, due to its contamination with lipid A-associated protein (Hogan and Vogel, 1987). After 16 hours of incubation with tumor targets, the uppermost 0.1 ml of the total 0.2 ml supernatant was removed and assayed for radioactivity in an automatic gamma spectrometer. Results are expressed as percent specific ^{51}Cr -release (percent cytotoxicity) and were calculated by the following formula:

$$\text{Percent Specific Cytotoxicity} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100$$

Total cpm were obtained from incubation of ^{51}Cr -labeled P815 with 200 μl of 0.5% sodium dodecyl sulfate. Spontaneous release from unstimulated monolayers was approximately 20-30% of the total cpm and was always less than or equal to the release from targets incubated in medium alone.

Statistical analysis was performed using a two-way analysis of variance (ANOVA) and the differences between treatment means determined by the least significant difference comparison (LSD; Snedecor and Cochran, 1977). Differences between treatment groups were taken to be statistically significant if $p \leq 0.05$.

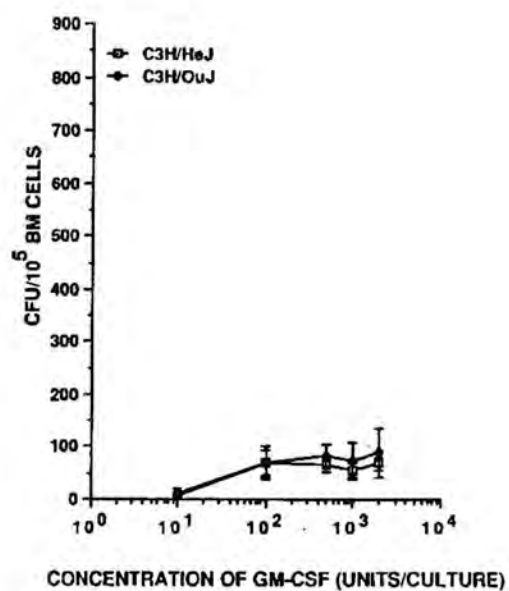
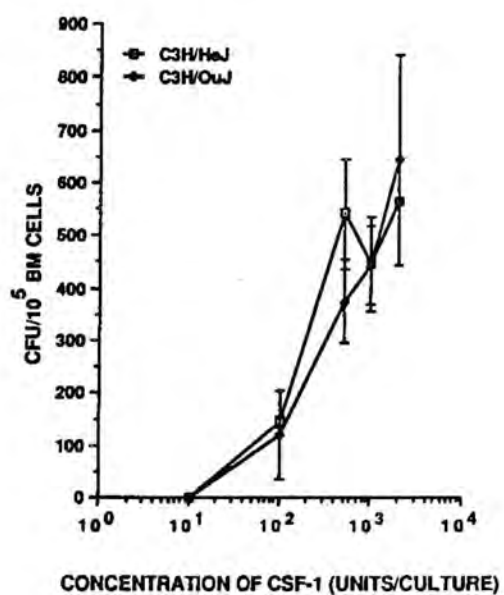
RESULTS

ANALYSIS OF THE RESPONSIVENESS OF BONE MARROW PROGENITORS IN SOFT AGAR TO GM-CSF VERSUS CSF-1

Both GM-CSF and CSF-1 have been shown to give rise to mature macrophages *in vitro* from bone marrow progenitors (reviewed by Metcalf, 1986). The goal of this dissertation was to examine the raison d'être for this apparent cytokine redundancy. To do this, highly purified or recombinant preparations of these two cytokines were obtained, and bone marrow progenitors examined for their responsiveness to these cytokines, using a variety of different functional criteria. Our initial studies focused on the question, "Are the numbers of bone marrow progenitors responsive to GM-CSF and CSF-1 equivalent in C3H mice?"

Quantitation of the number of bone marrow progenitors responsive to GM-CSF and CSF-1 using a soft-agar overlay assay. To compare the numbers of bone marrow progenitors (BMP) which respond to GM-CSF or CSF-1, bone marrow cells derived from C3H mouse strains were cultured over a broad range of CSF concentrations (10 - 2000 U/culture) in a double-agar overlay system. Since it had been reported previously that endotoxin-hyporesponsive (Lps^d) C3H/HeJ mice possess greater numbers of CSF-1-responsive progenitors than mouse strains which respond normally to endotoxin (Lps^n) (MacVittie and Weinberg, 1980), both C3H/HeJ (Lps^d) and C3H/OuJ (Lps^n) bone marrow cells were compared for responsiveness to murine rGM-CSF and nCSF-1. Figure 3 illustrates that at the end of the 10-day culture period, the number of BMP which responded to nCSF-1 to form colonies (≥ 50 cells) was approximately six-fold greater than the number which responded to rGM-CSF. Figure 3 also shows that there was no significant difference in the response of BMP from these two mouse strains to either of the two CSF preparations; each strain exhibited the same approximate difference in the number of nCSF-1- and rGM-CSF-responsive BMP on the dose plateau. In the presence of

Figure 3. Dose-response curve for C3H bone marrow progenitor colony formation in the presence of GM-CSF or CSF-1. Five $\times 10^4$ bone marrow cells were cultured in the double agar overlay assay (described in the Materials and Methods) in the presence of nCSF-1 (left) or rGM-CSF (right). At the end of the 10-day assay period, colonies (≥ 50 cells) were counted. The data represent the arithmetic mean \pm SEM for 4 - 10 individual experiments at each concentration of CSF tested.



nCSF-1, the number of BMP which responded to form colonies was relatively constant over a concentration range of 200 - 2000 U/culture and declined rapidly below 200 U/culture. In contrast, in response to rGM-CSF, maximal numbers of progenitors were maintained over a broader dose range (50 - 2000 U/culture). At concentrations as low as 10 U/culture, rGM-CSF was still able to supporting limited colony formation, while nCSF-1 was not. Although the data in Figure 3 were compiled using a natural preparation of murine CSF-1 exclusively, the numbers of progenitor cells which responded to various concentrations of HUrCSF-1 were not significantly different (i.e., at 10 U/culture, 100 U/culture, 1000 U/culture, and 2000 U/culture, we observed 0 , 116 ± 36 , 364 ± 5 , and 435 ± 9 CFU/ 10^5 input cells, respectively). The colonies which formed from BMP in the presence of rGM-CSF or nCSF-1 were consistently < 2 mm in diameter when measured on Day 10 (D10).

To ensure that the differences in colony formation observed at Day 10 with 5×10^4 cells per culture (Figure 3) were reflected over a wide range of input cell numbers, the number of bone marrow cells cultured in the presence of excess CSF (2000 U/culture) was varied and the cultures analyzed for colony formation at Day 10 (Table 1). In the presence of either nCSF-1 or rGM-CSF, there is a proportional decrease in the number of BMP which formed colonies with lowered input bone marrow cell concentrations. The relative differences observed in the number of BMP responsive to the two cytokines was maintained over a broad range of input cell numbers. Therefore, it is unlikely that the relatively low numbers of BMP which respond to rGM-CSF to form colonies are attributable to the activity of a suppressor cell population which is enriched and exerts its suppressive effects at high input cell numbers.

The kinetics of colony formation under the influence of rGM-CSF and nCSF-1 was investigated by examination of the cultures at various times over the 10 day culture period. Figure 4 demonstrates that when cultures were examined at Day 5, no significant difference was observed in the number of bone marrow-responsive cells for rGM-CSF versus the number which respond to nCSF-1. By Day 7, the number of rGM-CSF-responsive progenitors decreased slightly, while nCSF-1-responsive progenitor numbers increased. Analysis of cultures on Day 10 showed that the number of nCSF-1-specific colonies had continued to increase, resulting in

Table 1

Effect of Input Bone Marrow Cell Number on Colony Formation^a

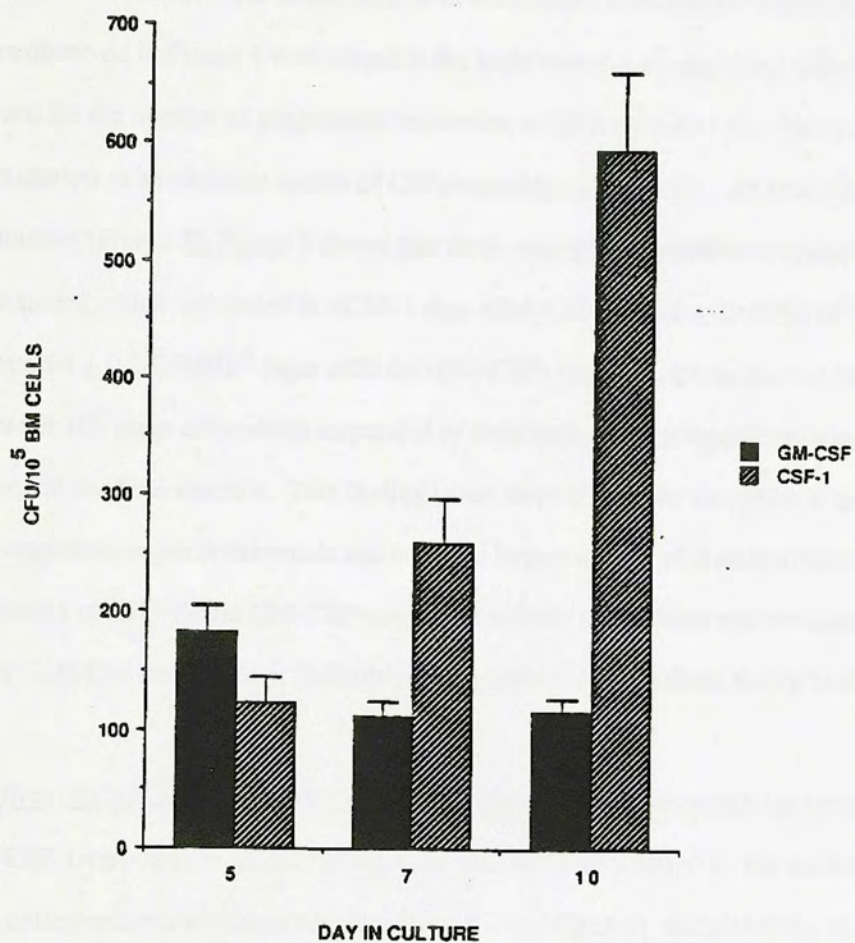
<u>INPUT CELL NUMBER</u>	<u>COLONIES/CULTURE^b</u>	
	<u>CSF-1-DERIVED</u>	<u>GM-CSF-DERIVED</u>
5.0×10^2	7 ± 0	NT ^c
5.0×10^3	37 ± 1	3 ± 1
2.5×10^4	173 ± 0	22 ± 5
5.0×10^4	373 ± 57	37 ± 5
1.0×10^5	NT	110 ± 22
5.0×10^5	NT	338 ± 21

^a Various input numbers of bone marrow cells were cultured in the presence of an excess of nCSF-1 or rGM-CSF (2000 U/culture) and assayed at Day 10 for colony formation.

^b The data represent the arithmetic mean \pm standard deviation from a representative experiment in which duplicate cultures were examined.

^c NT represents conditions which were not tested.

Figure 4. Time course for the development of colony formation in the presence of CSF-1 or GM-CSF. Five $\times 10^4$ bone marrow cells were cultured in the double agar overlay assay in an excess (2000 U/culture) of nCSF-1 or rGM-CSF. At various times during the 10 day culture period, cultures were examined for colony formation (≥ 25 colonies at Day 5 and ≥ 50 colonies on Days 7 and 10). The data represent the arithmetic mean \pm SEM for 5 individual experiments.



the large difference between rGM-CSF and nCSF-1 progenitor numbers which was originally illustrated in Figure 3.

Analysis of the number of splenic progenitors responsive to GM-CSF and CSF-1. To determine whether the difference in the number of rGM-CSF- versus nCSF-1-responsive progenitors observed in Figure 3 was unique to the bone marrow, an additional organ system was analyzed for the number of progenitors responsive to each cytokine. For this study, the spleen was chosen as an alternate source of CSF-responsive progenitors. As was observed in the bone marrow (Figure 3), Figure 5 shows that there were greater numbers of progenitor cells in the spleen which responded to nCSF-1 than rGM-CSF; i.e., 53 ± 10 CFU/ 10^5 input cells for nCSF-1 versus 4 ± 0.5 CFU/ 10^5 input cells for rGM-CSF; however, the number of splenic progenitors per 10^5 input cells which responded to form colonies was significantly less than that observed in the bone marrow. This finding is not surprising since the spleen is not the major hematopoietic organ in mammals and contains large numbers of mature effector cells. This distribution of CSF-1- and GM-CSF-responsive splenic progenitors was the same using C3H/HeJ or C3H/OuJ mice. These findings are also consistent with those shown in Figure 3.

Effect of CSF deprivation on colony formation. To examine further the increase in the number of CSF-1-responsive colonies which form with time in culture (i.e., the marked increase in colony number observed between Days 5 - 10; Figure 4), the sensitivity of progenitors to CSF deprivation early in the culture period was examined to determine if a population of CSF-1-responsive progenitors exists which can be stimulated at a later time in culture to form colonies by Day 10. This possibility is supported by previous findings of Lin (1974) and MacVittie and McCarthy (1977) which demonstrated that in the peritoneal cavity and in the lymph node, there exist cells which respond to CSF-1 to form colonies after a significant "lag time" (7 - 14 days) in culture. To this end, cells were cultured on Day 0 and were overlaid with CSF at various times during the first 7 days of culture (Figure 6A). All cultures were examined 10 days after the initial seeding of bone marrow cells (i.e., on D10).

Figure 5. Quantitation of the number of progenitors responsive to GM-CSF and CSF-1 in the spleen. Five $\times 10^5$ LSM-purified spleen cells were cultured in the double agar overlay assay in an excess (2000 U/culture) of nCSF-1 or rGM-CSF. The number of CSF-1- and rGM-CSF-responsive progenitors in the spleen were examined by colony formation (≥ 50 cells) following 10 days in culture at 37⁰ C and 5% CO₂. The data represent the arithmetic mean \pm SEM for n = 3 individual experiments using C3H/HeJ mice and n = 4 individual experiments using C3H/OuJ mice.

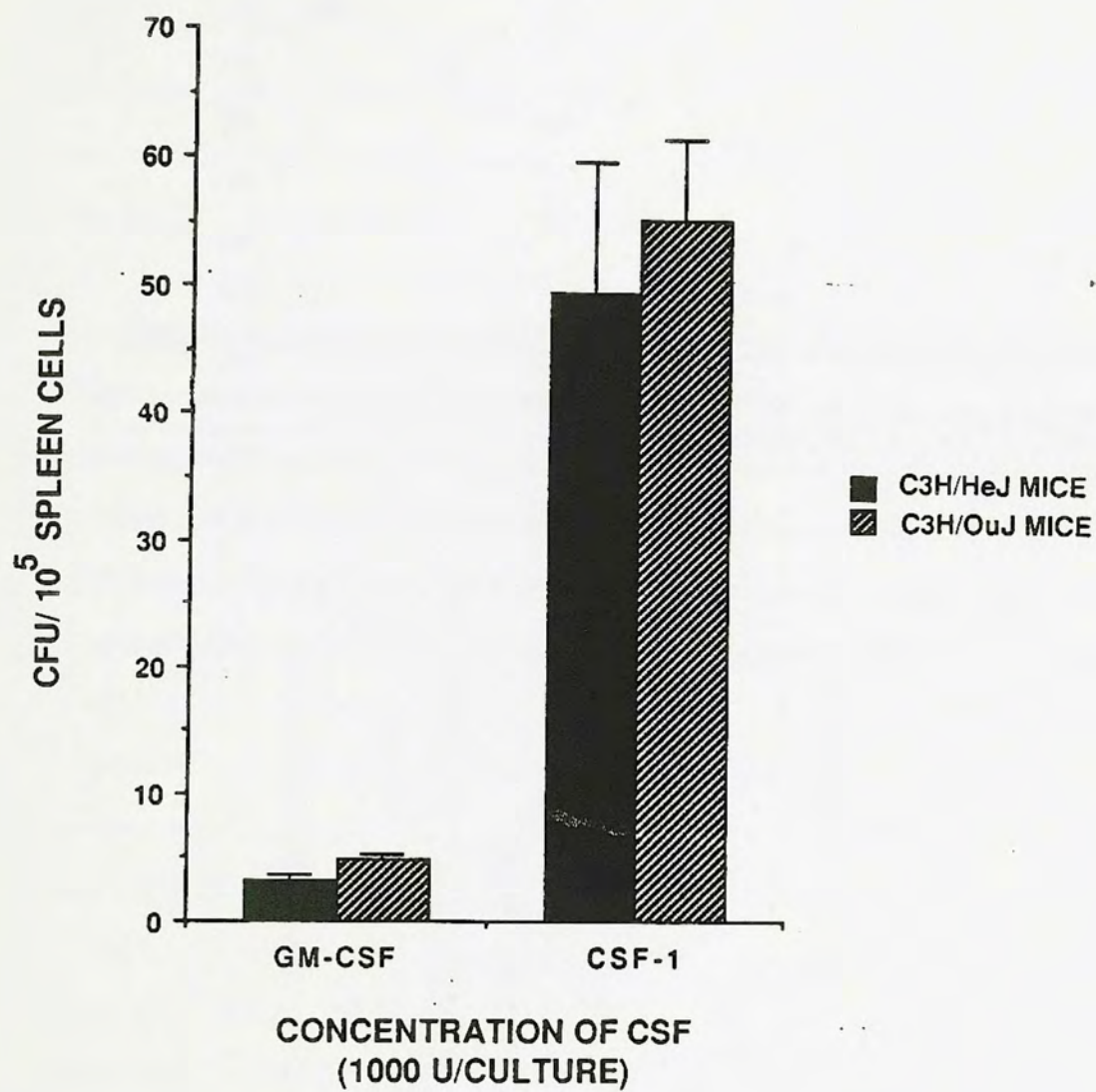


Figure 6. Determination of the number of CSF-responsive progenitor cells following CSF deprivation in soft agar. Five $\times 10^4$ LSM-purified bone marrow cells were cultured on D0 in the presence of 2000 U/culture of rGM-CSF, nCSF-1, or medium alone. Cultures which received medium on D0 were then overlayed with 2000 U/ml of either nCSF-1 or rGM-CSF at various times during the culture period (panel A). Colony formation was examined ten days from the initiation of cultures (D0). The data (panel B) represent the arithmetic mean \pm SEM for $n = 6$ separate experiments.

A.

PROTOCOL

D0 n cells n cells n cells n cells n cells n cells

D0 CSF

D1 CSF

D2 CSF

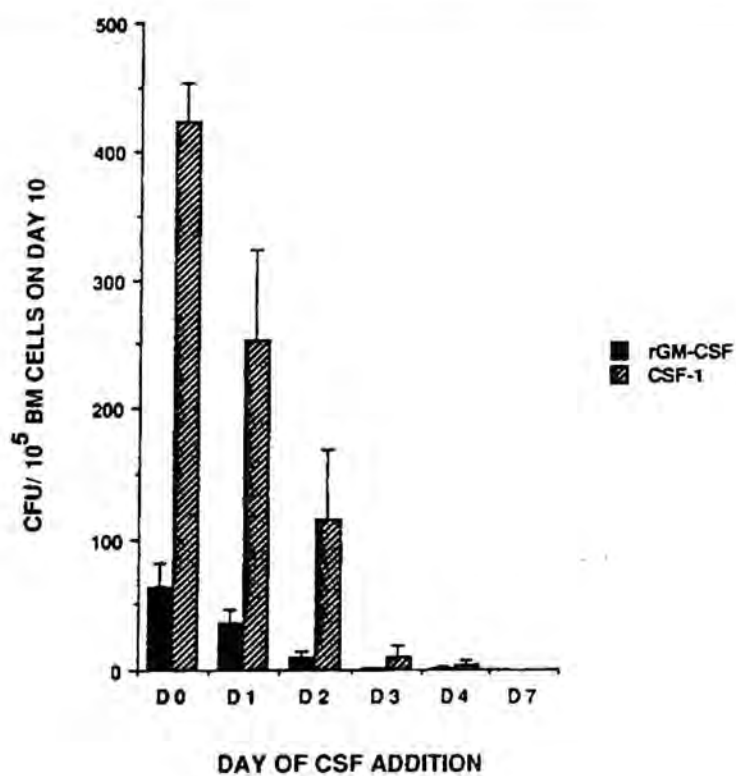
D3 CSF

D4 CSF

D7 CSF

D10 COUNT ALL PROGENITORS

B.



The data in Figure 6B illustrate that colony-forming ability was reduced significantly in both nCSF-1 and rGM-CSF cultures following a 24-hour period of CSF deprivation, such that by 3 - 4 days of CSF-deprivation, little colony formation was observed. Examination of CSF-deprived cultures for colony formation 10 days after the day of addition of the CSF (rather than on D10 of culture; Figure 7A) revealed that the colony-forming ability of cultures which had been CSF-deprived for as little as 24 hours was markedly reduced (Figure 7B). Thus, at D10, as well as 10 days after the addition of CSF, the number of colonies which formed after CSF-deprivation was less than that observed in cultures which received CSF on D0 of culture. Therefore, the differences in progenitor responsiveness to CSF-1 exhibited between Days 5 and 10 of culture (Figure 4) could not be attributed to a subpopulation of CSF-1-responsive progenitors which responds to CSF-1 later in culture to form full-sized colonies (≥ 50 cells) between Days 5 and 10.

An alternate explanation for the differences in colony formation observed between CSF-1- and GM-CSF cultures at D10 (Figure 3) could be that CSF-1 and GM-CSF differ in their stability over the 10 day culture period. To determine if the failure to increase colony formation between D5 and D10 in GM-CSF-stimulated cultures were due to instability of GM-CSF with time in culture, GM-CSF-initiated cultures were supplemented with an additional dose of GM-CSF on D5 and colonies counted on D10. If degradation of GM-CSF resulted in limited colony formation, a GM-CSF supplement on Day 5 might overcome the deficit and result in additional colony formation, as seen in CSF-1 cultures (Figure 4). However, this was not the case. Cultures which received an additional supplement of 2000 U/culture of GM-CSF did not exhibit colony numbers which were any greater than the numbers seen in cultures which received 2000 U on D0 (i.e., 72 ± 0 CFU/ 10^5 input cells in cultures which received GM-CSF on D0 only versus 72 ± 4 CFU/ 10^5 input cells in cultures supplemented on D5).

From the data shown above, it is clear that there are greater numbers of progenitors in both the bone marrow and spleen which respond to CSF-1 than to GM-CSF to form colonies by Day 10. No differences were observed in progenitor responsiveness between C3H/HeJ and C3H/OuJ mouse strains.

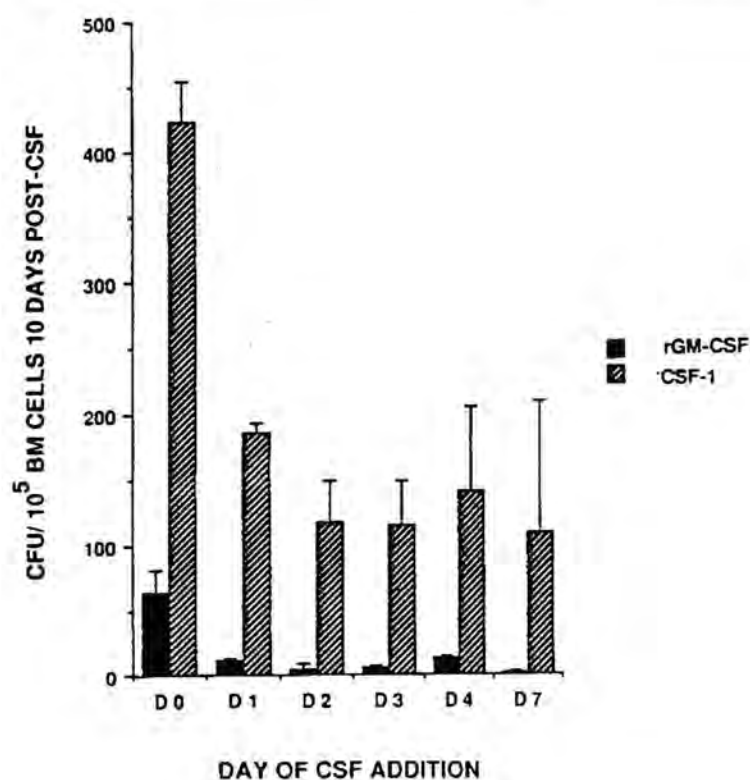
Figure 7. Examination of colony formation in soft agar 10 days from CSF addition in CSF-deprived cultures. Five $\times 10^4$ LSM-purified bone marrow cells were cultured in the presence of 2000 U/culture of rGM-CSF, nCSF-1, or medium alone (panel A) as described in Figure 6. Colony formation was examined 10 days after the day of addition of CSF to cultures. The data (panel B) represent the arithmetic mean \pm SEM for $n = 4$ separate experiments.

A.

PROTOCOL

	n cells	n cells	n cells	n cells	n cells	n cells
D0	CSF					
D1		CSF				
D2			CSF			
D3				CSF		
D4					CSF	
D7						CSF
D10	COUNT					
D11		COUNT				
D12			COUNT			
D13				COUNT		
D14					COUNT	
D17						COUNT

B.



ANALYSIS OF THE RESPONSIVENESS OF BONE MARROW PROGENITORS TO GM-CSF AND CSF-1 IN LIQUID CULTURE

To ensure that the differences observed in the numbers of bone marrow progenitors responsive to GM-CSF versus CSF-1 were not unique to the agar-overlay system employed or due to differences in diffusion capabilities of the two CSFs in agar, the yield of mature macrophages produced in liquid culture under the influence of GM-CSF or CSF-1 was next examined. This established optimal liquid culture conditions for the proliferative expansion of progenitors. These culture techniques enabled the further examination of mature bone marrow-derived macrophages which developed under the influence of either GM-CSF or CSF-1 with respect to a variety of morphological and differentiative characteristics.

Analysis of progenitor responsiveness to GM-CSF and CSF-1 in liquid culture. Figure 8 illustrates that over a wide dose range of GM-CSF or CSF-1, differences in macrophage yield in liquid culture paralleled those observed in the soft agar progenitor assay, i.e., the number of rGM-CSF-derived macrophages was less than that observed in the nCSF-1-derived cultures. Cultures grown in an excess of CSF (1000 U/ml) resulted in an approximate five-fold difference in yield. This difference could be minimized by culturing the macrophages in a minimal concentration of CSF (250 U/ml; Figure 8) and by increasing the number of bone marrow cells initially seeded in rGM-CSF cultures from 1×10^7 to 3×10^7 per flask (Table 2). These conditions (250 U/ml CSF and 1×10^7 input BMP for CSF-1 versus 3×10^7 input BMP for GM-CSF) were utilized in all subsequent experiments for the generation of mature macrophages in liquid culture and allowed for nearly equivalent yields of macrophages for additional experimental analyses.

Morphological characteristics of macrophages grown in liquid culture. Progenitor cells grown for 7 days in liquid culture in the presence of rGM-CSF or nCSF-1 exhibited both

Figure 8. Yield of bone marrow-derived macrophages in response to various concentrations of CSF-1 or GM-CSF in liquid culture. C3H/HeJ bone marrow cells were seeded at 1×10^7 for CSF-1 (O) or 3×10^7 for GM-CSF (O) cultures. Following 7 days in liquid culture, the number of adherent macrophages was determined and the yield expressed on the basis of 1×10^7 input (seeded) bone marrow cells. Each data point represents the yield derived from an individual experiment with $n = 3$ for rGM-CSF, $n = 4 - 7$ for nGM-CSF, and $n = 6 - 10$ for nCSF-1.

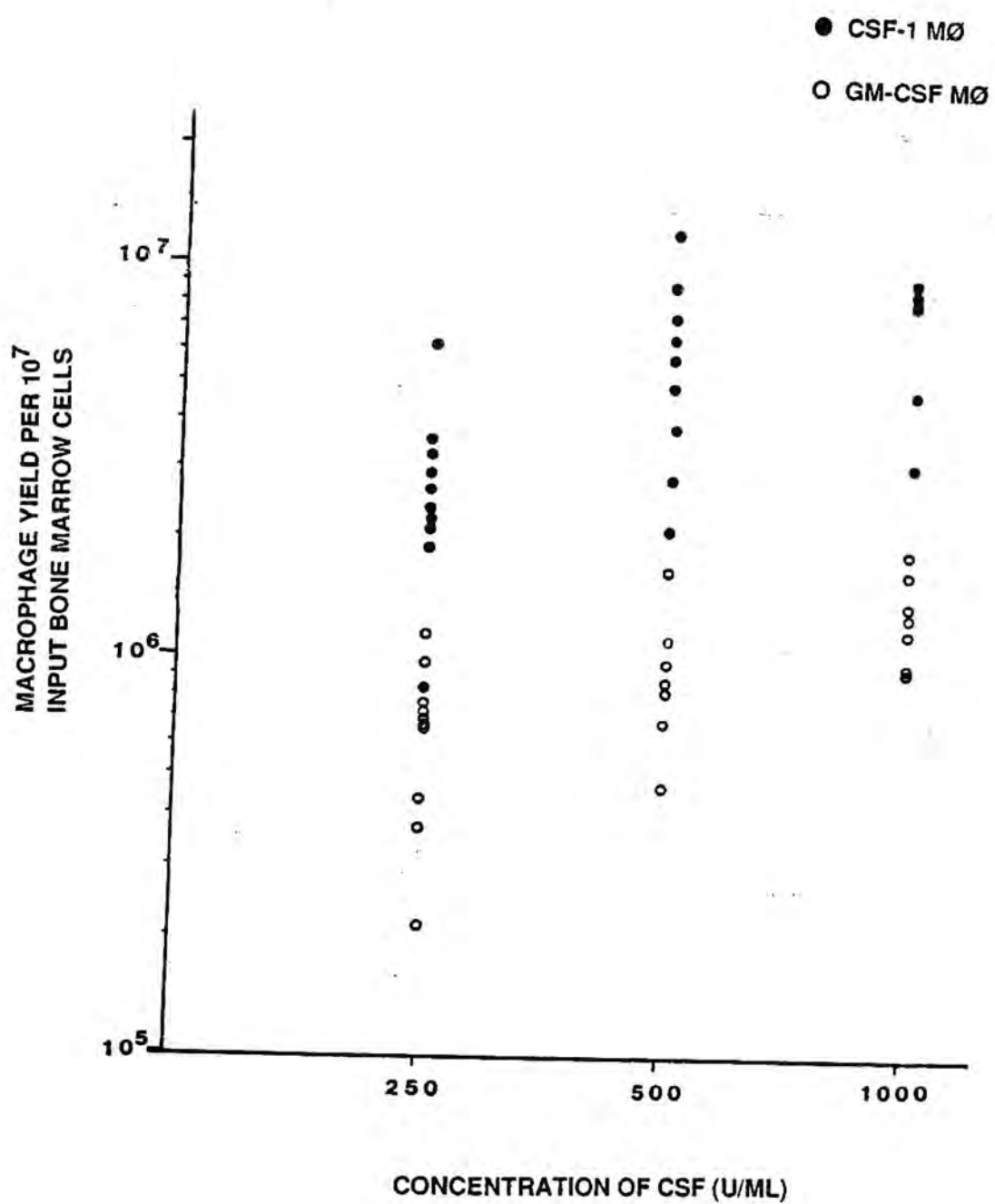


Table 2

Comparison of Macrophage Yield Derived from Bone Marrow Progenitors under
the Influence of CSF-1 or GM-CSF^a

<u>CSF SOURCE</u>	<u>NUMBER OF BONE MARROW CELLS SEEDED/FLASK</u>	<u>YIELD/FLASK^b (95% CONFIDENCE LIMITS)</u>
CSF-1 (250 U/ML)	1×10^7	3.4×10^6 ($2.3 - 5.0 \times 10^6$)
GM-CSF (250 U/ML)	3×10^7	1.9×10^6 ($1.1 - 3.2 \times 10^6$)

^a Bone marrow cells were plated at 1×10^7 /flask (for CSF-1) and 3×10^7 /flask for (GM-CSF), and were supplemented for 7 days as described in the Materials and Methods.

^b The yields of mature macrophages which resulted from culture in nCSF-1 or rGM-CSF were determined after Dispase II treatment of 7 day cultures. The data represent the geometric means and the 95% confidence limits for $n = 12$ individual experiments for nCSF-1 and $n = 9$ individual experiments for rGM-CSF.

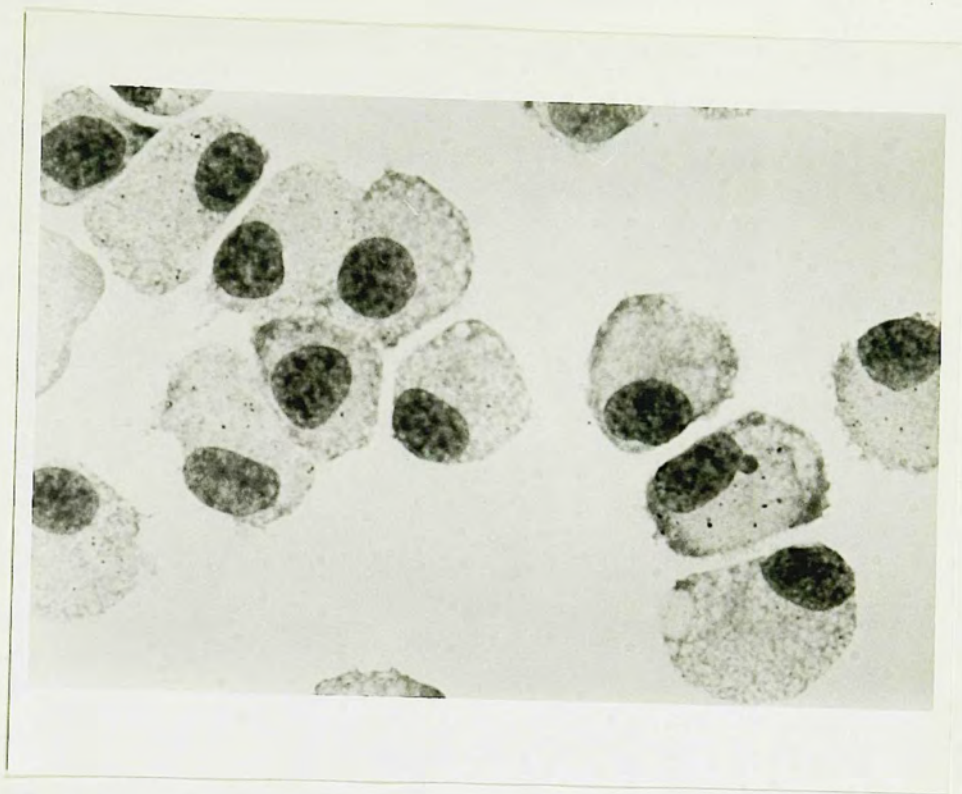
similarities and differences with regard to their morphological characteristics. Although the adherent cells derived under the influence of either rGM-CSF or nCSF-1 in liquid culture were 100% mononuclear in nature, as determined by examination of cytocentrifuged, Wright-stained smears (Figure 9), microscopic examination of macrophages propagated in liquid cultures also showed differences with respect to their size heterogeneity and degree of vacuolization: nCSF-1-derived cells (Figure 9A) were more uniform in size, while rGM-CSF-derived (Figure 9B) macrophages exhibited greater size heterogeneity. In addition, rGM-CSF-derived macrophages were more vacuolated than CSF-1-derived macrophages. To quantify these visual differences in cell size, macrophages from nCSF-1 and rGM-CSF cultures were subjected to cell sizing analysis through the use of the Coulter Channelyzer and were examined at various times during the 7-day culture period (Figure 10). There were no differences in the cell sizing profiles for rGM-CSF and nCSF-1 through Day 2 (D2). However, with additional time in culture, the nCSF-1-derived cultures exhibited a reduction in the number of small cells such that, by Day 7 (D7), the majority of cells fell within a single major peak of larger cells with an average diameter of 12.2 μ (Figure 10). Cell sizing profiles of rGM-CSF cultures also showed an increase in the number of large cells by Day 3. In contrast, rGM-CSF-derived cells, by D7, exhibited a broader distribution in the average cell size (ranging from 8.3 - 11.4 μ diameter; Figure 10). Extension of the liquid culture period to Day 10 did not alter the cell sizing profile of rGM-CSF-derived macrophages observed at D7 and did not result in a cell sizing profile similar to that of nCSF-1-derived cells. It should be noted, that cell sizing profile of mature (D7) macrophages cultured in nCSF-1 (Figure 10) was indistinguishable from that of cells grown in the presence of HU-CSF-1 (Figure 11). In addition, Table 3 demonstrates that the cell sizing profile differences observed between nCSF-1- and rGM-CSF-derived macrophages (Figure 10) were not unique to the C3H/HeJ mouse strain. A similar distribution of cell sizes was observed in mature (D7), C3H/OuJ bone marrow-derived macrophages following culture in the presence of nCSF-1 or rGM-CSF.

These data indicate that the differences in number of progenitors responsive to CSF-1 versus GM-CSF observed in soft agar were not unique to that culture system, but rather, were

Figure 9. Morphological characteristics of CSF-1 and GM-CSF-derived macrophages.

Macrophages grown in liquid culture for 7 days in the presence of nCSF-1 (A) or rGM-CSF (B) were harvested, and the resulting cells were cytocentrifuged, fixed, and stained as described in the Materials and Methods prior to examination under oil immersion (1000x).

A.



B.

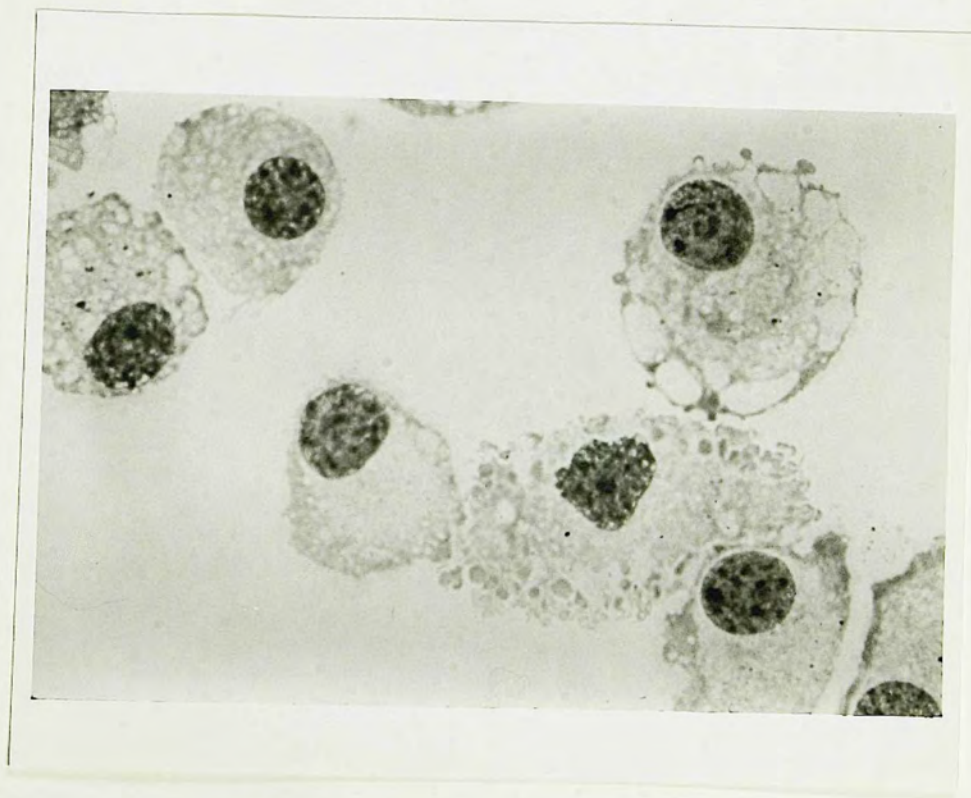


Figure 10. Cell sizing profiles of CSF-1 and GM-CSF-derived macrophages over the 7-day liquid culture period. Recombinant GM-CSF- (left panel) and nCSF-1-derived macrophages (right panel) were enzymatically removed and cell sizing profiles determined at various times during the 7-day culture period using Coulter Channelyzer analysis. Approximately 10,000 - 14,000 cells were analyzed using the Coulter Channelyzer settings of attenuation = 4, window width = 70, and matching = 10.

Figure 11. Cell sizing profile of mature (D7) HUrCSF-1-derived macrophages.

HUrCSF-1-derived macrophages were enzymatically removed after 7 days in liquid culture and cell sizing profiles determined using Coulter Channelyzer analysis as described in Figure 10.

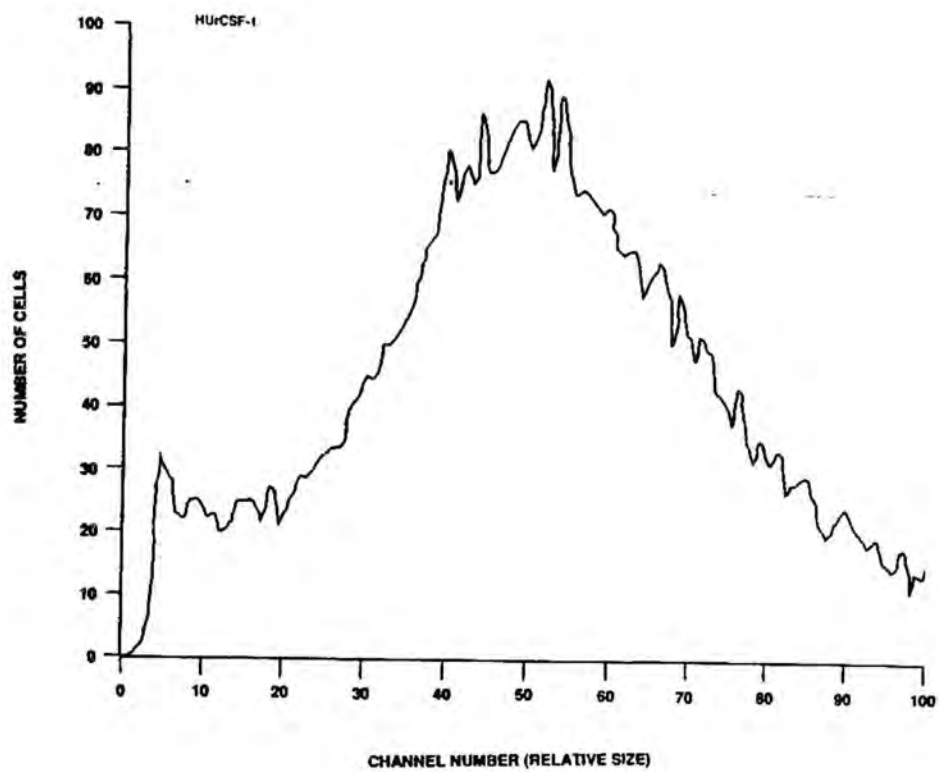


Table 3

Cell size distribution of C3H/HeJ and C3H/OuJ mature macrophages derived in
GM-CSF or CSF-1^a

<u>CELL TYPE</u>	<u>CHANNEL NUMBER^b</u>		
	<u>0 - 15</u>	<u>16 - 45</u>	<u>46 - 99</u>
<u>GM-CSF</u>			
C3H/HeJ	35.7	41.8	22.5
C3H/OuJ	40.3	47.6	12.1
<u>CSF-1</u>			
C3H/HeJ	7.4	36.9	55.6
C3H/OuJ	10.5	43.6	45.9

^a Bone marrow progenitors derived from C3H/HeJ or C3H/OuJ mice were cultured in liquid culture in the presence of 250 U/ml of either rGM-CSF or nCSF-1 as described in the Materials and Methods. Following the standard 7 day culture period, mature macrophages were removed enzymatically and the cell size distribution determined using Coulter Channelyzer analysis.

^b The data represent the percentage of cells analyzed which are between channel numbers 0 - 15 (7.0-9.3 μ), 16 - 45 (9.4-11.9 μ), and 46 - 99 (12.1-15.0 μ). The data were derived from a single representative experiment of 4 separate experiments in which 10,000 to 14,000 cells were analyzed per treatment.

also reflected in the yields of mature macrophages when liquid culture techniques were utilized. Macrophages derived in liquid culture under the influence of GM-CSF or CSF-1 also exhibited differences in their morphologies and cell size distributions.

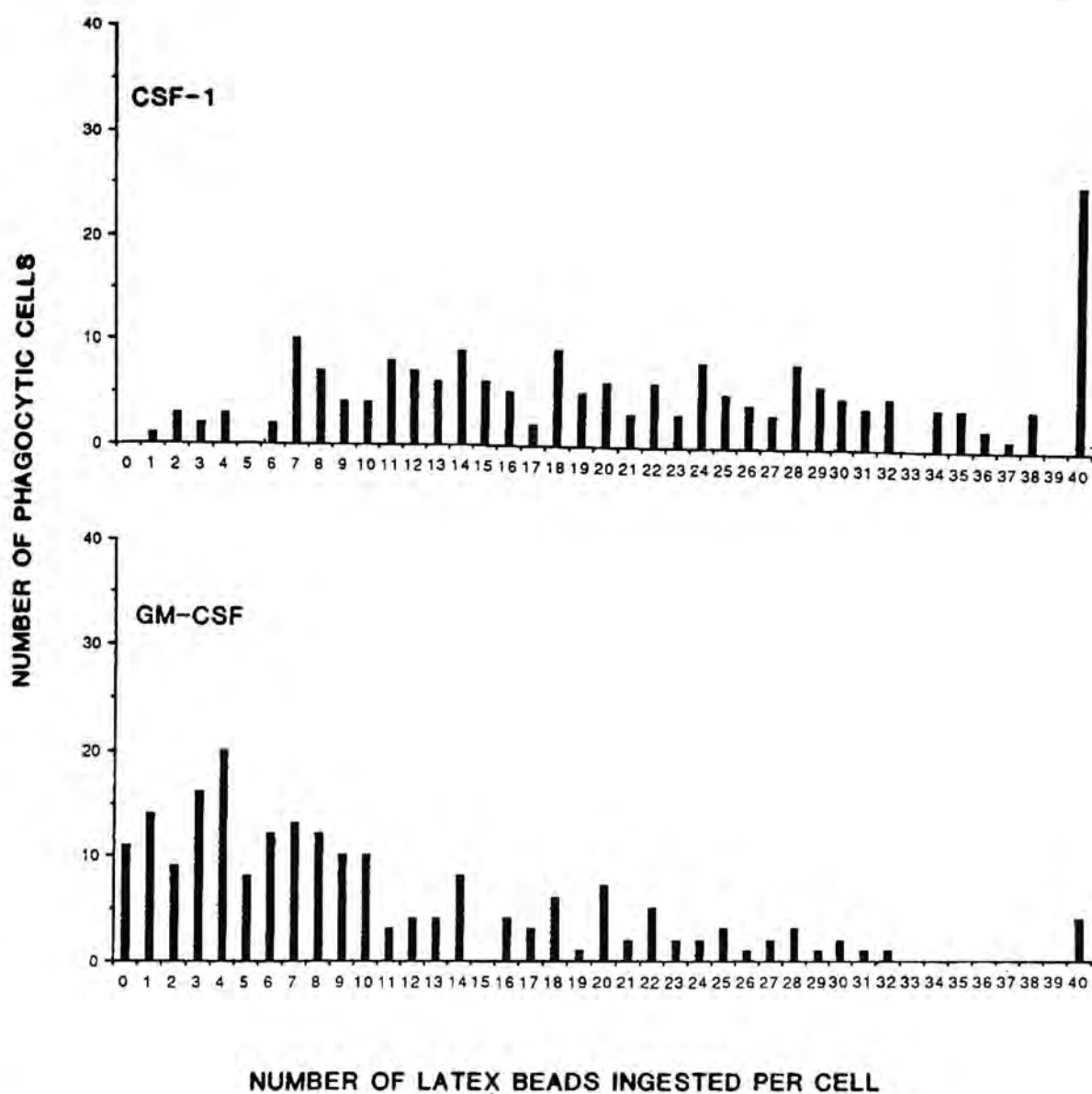
FUNCTIONAL CHARACTERISTICS OF MATURE (DAY 7) MACROPHAGES

The ability to culture mature macrophages from bone marrow progenitors under controlled conditions, i.e., following stimulation of BMP by a single species of exogenous, highly purified or recombinant CSF, allowed us to ask a variety of questions about the state of differentiation exhibited by the resultant, D7 macrophages. Previous studies have demonstrated that as macrophages become more differentiated there is an increase in cell size and vacuolization (Cohn, 1978; Rosenstreich, 1981). The morphological differences related to size and vacuolization which were observed between CSF-1- and GM-CSF-derived macrophages prompted us to examine these mature bone marrow-derived macrophages with respect to their functional and differentiative characteristics. Based on the model of linear acquisition of specific morphological and functional "hallmarks" during differentiation (Figure 1), we proceeded by comparing these two populations for a variety of functional markers.

Analysis of the ability of CSF-1- and GM-CSF-derived macrophages to phagocytose latex beads. One early functional and differentiative characteristic, non-receptor-mediated phagocytosis (latex ingestion), was compared in mature macrophages derived from nCSF-1- and nGM-CSF-treated BMP cultures. Figure 12 demonstrates that both populations could phagocytose latex beads: nCSF-1-derived macrophages were 100% phagocytic and had a weighted mean of 22 beads ingested per cell, whereas nGM-CSF-derived cells were > 95% phagocytic but exhibited a weighted mean of only 11 beads ingested per cell. These data, which were obtained using highly purified murine CSF-1 and GM-CSF preparations, were subsequently confirmed using HUrCSF-1-derived macrophages and rGM-CSF-derived macrophages, i.e., HUrCSF-1 macrophages were 100% positive for latex ingestion and exhibited

Figure 12. Phagocytic capabilities of CSF-1- and GM-CSF-derived macrophages for latex beads.

CSF-1-(top) and nGM-CSF-(bottom) derived macrophages were harvested following the 7-day liquid culture period and were plated at 2×10^5 cells per chamber in 8-well Lab-Tek chamber slides. Following a 4 hour adherence step, cultures were allowed to ingest latex beads for 1 hour at 37° C. Following this ingestion period, cultures were washed free of non-ingested latex beads, fixed, stained, and scored under oil immersion for the number of latex beads per cell. Data were derived from one of 2 separate experiments in which 200 cells per CSF treatment were scored.

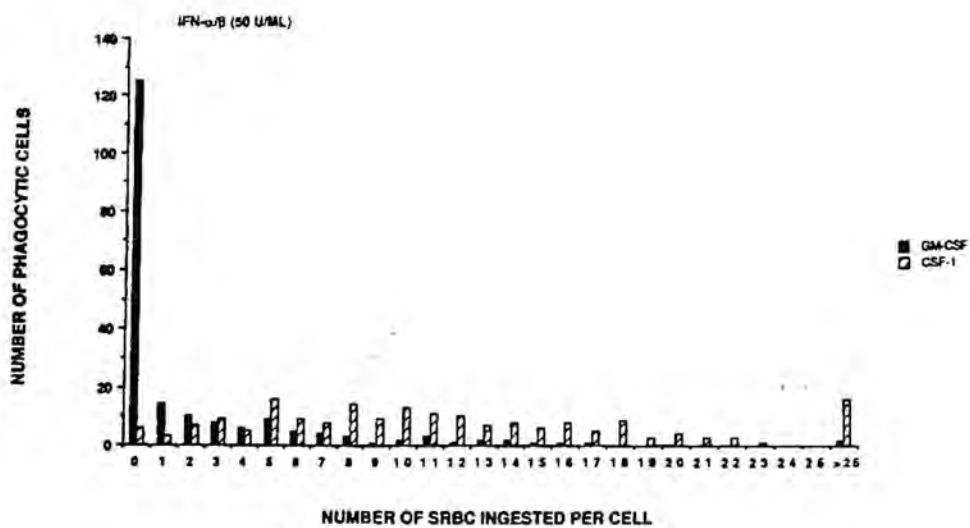
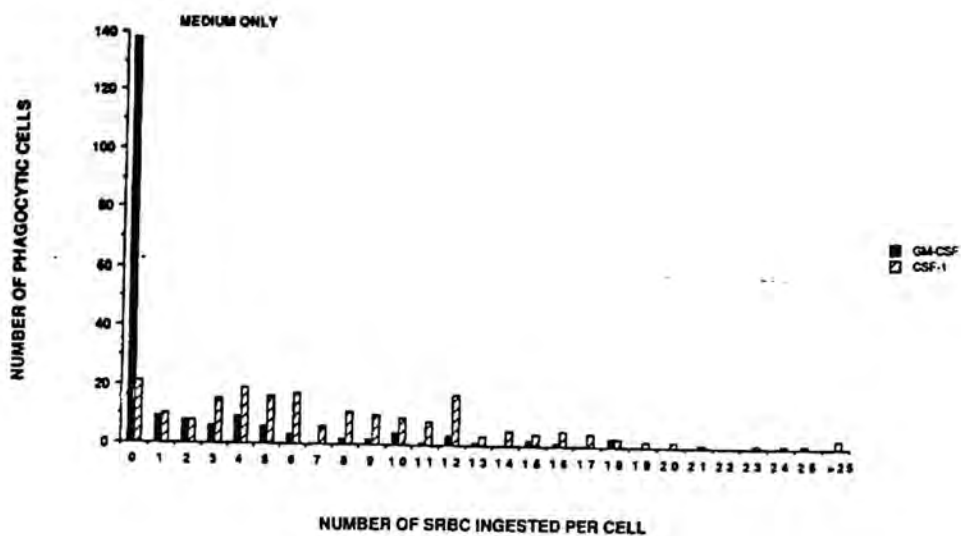


a weighted mean of 24 ingested beads per cell and rGM-CSF macrophages were 91% positive for latex ingestion and had a weighted mean of 16 beads ingested per cell.

Analysis of Fc receptor-mediated phagocytosis and binding. In addition to non-specific phagocytosis of latex beads, examination of Fc receptor-mediated phagocytosis revealed an even more dramatic difference in the phagocytic capabilities of these two macrophage populations. Figure 13 (top panel) shows that approximately 90% of nCSF-1-derived macrophages were capable of ingesting opsonized SRBC (weighted mean of 8.5 IgG-coated SRBC ingested per phagocytic cell). Once again, macrophage cultures derived under the influence of HU-CSF-1 confirmed results obtained using the nCSF-1 preparation: 87% of the macrophages were positive for ingestion of opsonized SRBC and exhibited a weighted mean of 7.9 ingested IgG-coated SRBC per phagocytic cell. In contrast, only 30% of the rGM-CSF macrophages were capable of Fc receptor-mediated phagocytosis (weighted mean of 6.2 IgG-coated SRBC ingested per phagocytic cell).

Due to the relatively low phagocytic capacity of rGM-CSF-derived macrophages with regard to non-specific (latex) and specific (Fc-mediated) phagocytosis, we next sought to determine if the difference in Fc-mediated phagocytosis observed between the two macrophage populations were due to a relative paucity in the number of Fc receptors on GM-CSF-derived macrophages or due solely to an inherent deficiency in their ability to ingest particles. To examine this question, macrophages derived under the influence of either rGM-CSF or HU-CSF-1 were cultured for analysis of Fc receptor-mediated binding of opsonized erythrocytes. The presence of iodoacetic acid (IAA) in the opsonized SRBC preparation allows for the binding of IgG-coated SRBC, but has been shown to inhibit Fc receptor-mediated phagocytosis by > 95% (Walker and Demus, 1975; Vogel and Rosenstreich, 1979). Therefore, this technique allows for a measure of relative Fc receptor-mediated binding capacity. In the presence of IAA, 69% of the rGM-CSF-derived macrophages bound opsonized SRBC (weighted mean of 8.1 SRBC bound per positive cell), but this level was far below that observed in HU-CSF-1-derived macrophages (89%; weighted mean of 12.1 SRBC bound per positive cell).

Figure 13. Fc-receptor-mediated phagocytic capabilities of CSF-1- and GM-CSF-derived macrophages. Bone marrow-derived macrophages grown in the presence of nCSF-1 or rGM-CSF were harvested and plated at 2×10^5 cells per chamber in 8-well Lab-Tek chamber slides. Cultures were treated with medium (top) or 50 U/ml IFN- α/β (bottom) for 48 hours. Following this incubation period, culture supernatants were removed and replaced with 0.2 ml of opsonized SRBC and macrophages were allowed to phagocytose for 1 hour at 37° C. Following this 1 hour incubation, non-internalized SRBC were lysed and the monolayer was fixed, stained, and scored under oil immersion for the number of SRBC ingested. Data were derived from a single experiment, representative of 3 separate experiments, in which 200 cells per treatment were scored.



Thus, the reduced capacity of rGM-CSF-derived macrophages to phagocytose via the Fc receptor may also, in part, be due to a decreased capacity to bind opsonized SRBC prior to their ingestion.

The ability of IFN- α/β to increase Fc-mediated phagocytosis in C3H/HeJ macrophages has been well documented (Vogel and Fertsch, 1984; Fertsch and Vogel, 1984; Warren and Vogel, 1985a; and Warren and Vogel, 1985b). Therefore, the ability of these two populations of macrophages to respond to the inductive effects of IFN- α/β was also examined. Following a 48-hour incubation period in the presence IFN- α/β , rGM-CSF- and either nCSF-1- or HUrCSF-1-derived macrophages were examined for their Fc-mediated phagocytic capacities. Figure 13 (bottom panel) shows both nCSF-1- and rGM-CSF-derived macrophages exhibited an increase in the number of cells which were capable of ingesting opsonized SRBC following treatment with IFN- α/β . Macrophages derived under the influence of nCSF-1 exhibited a slight increase in their already high phagocytic capacity from 90% to 97% cells positive for ingestion of opsonized SRBC with an increase from 8.5 to 11.2 IgG-coated SRBC ingested per positive cell. These findings were confirmed using HUrCSF-1-derived macrophages which exhibited an increase from 87% to 98% cells positive for SRBC ingestion and an increase in the number of ingested SRBC per cell from 7.9 to 11.5 following IFN- α/β treatment. Recombinant GM-CSF-derived macrophages responded to IFN- α/β with a slight increase in phagocytic capability, from 30% to 38% cells positive for SRBC ingestion; however, no increase in the number of IgG-coated SRBC ingested per cell was observed following IFN- α/β treatment (6.2 SRBC ingested per positive cell in the presence of medium alone and 6.0 SRBC ingested per positive cell in the presence of IFN- α/β).

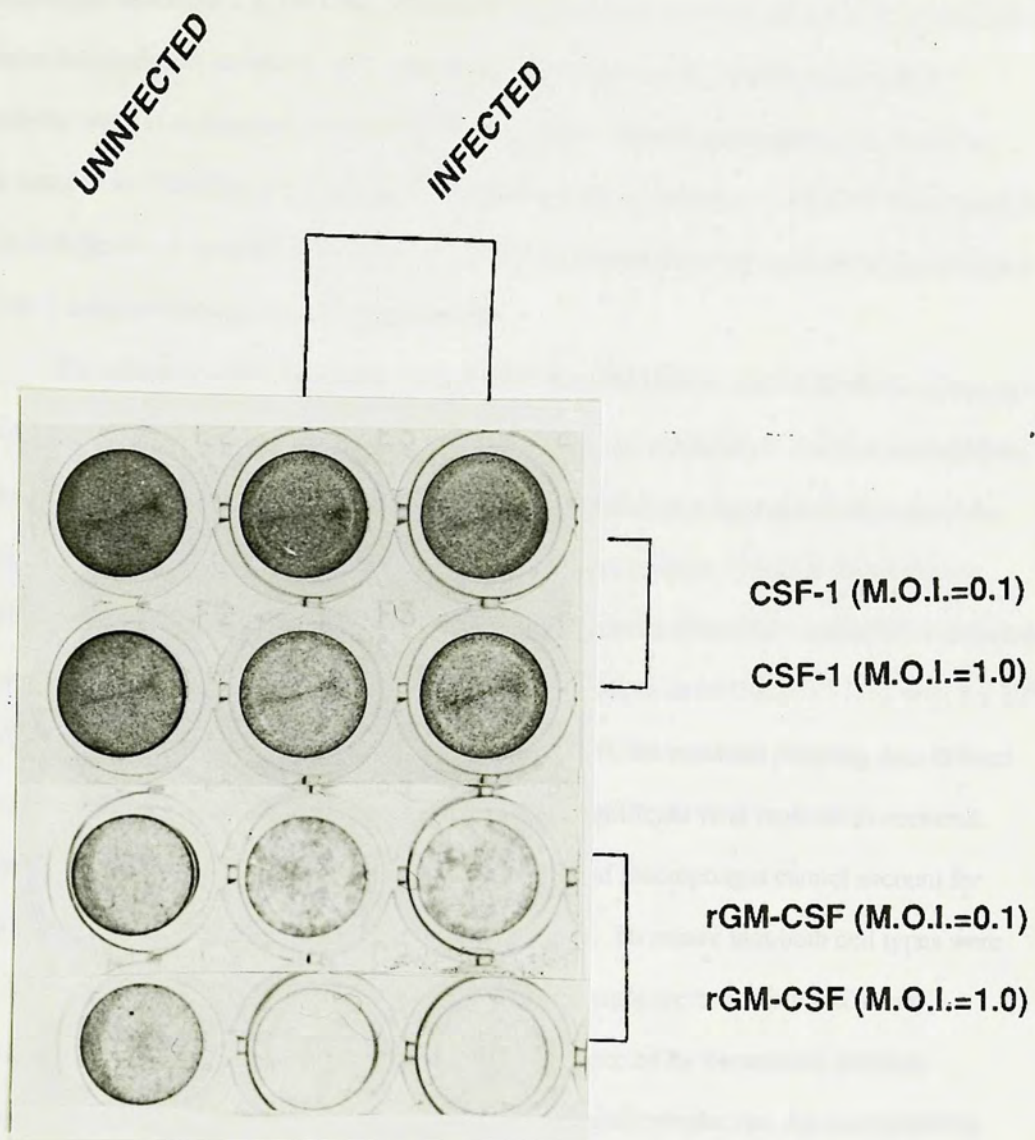
Thus, these experiments demonstrated that CSF-1-derived macrophages exhibit greater non-specific, as well as, Fc receptor-mediated phagocytic capabilities than GM-CSF-derived macrophages. These findings provided the first indication that bone marrow progenitors cultured in the presence of CSF-1 versus GM-CSF give rise to mature populations of macrophages which differ functionally.

Previous findings have shown that increased Fc receptor-mediated phagocytosis is mediated by endogenous interferon production by macrophages (Moore *et al.*, 1984; Warren and Vogel, 1985a). Thus, the demonstration of different Fc receptor-mediated phagocytic capabilities in CSF-1- versus GM-CSF-derived macrophages prompted us to examine the possible role of endogenous IFN- α/β production in these two macrophage populations. In the following experiments, CSF-1- and rGM-CSF-derived macrophages were analyzed for their ability to produce endogenous interferon.

Sensitivity of bone marrow-derived macrophages to vesicular stomatitis virus (VSV).

Interferons are probably best recognized for their capacity to mediate an antiviral state in interferon-sensitive cell types (reviewed by Gresser, 1984). Previous studies have shown a correlation between the ability of macrophages to phagocytose via their Fc receptors and the capacity of macrophages to be infected with VSV (Vogel and Fertsch, 1987), and both functions have been related to the production of endogenous IFN- α/β by macrophages (Moore, *et al.* 1984; Warren and Vogel, 1985a; Vogel and Fertsch, 1984; Gresser, 1984). Therefore, we next compared the ability of the CSF-1- and GM-CSF-derived macrophages to resist viral infection. Figure 14 shows that C3H/HeJ macrophages derived from BMP under the influence of highly purified, nCSF-1, were resistant to the cytopathic effects (CPE) of VSV when infected at an M.O.I. of 0.1. When the M.O.I. was increased ten-fold, nCSF-1-derived macrophages exhibited only minimal sensitivity. By comparison, rGM-CSF-derived macrophages were much more sensitive to VSV-induced CPE at both an M.O.I. of 0.1 and 1.0. To quantitate the CPE observed in bone marrow-derived macrophage cultures, uninfected monolayers were compared to infected cultures and the percent CPE determined by the amount of crystal violet stain taken up by the remaining intact cells (Vogel *et al.*, 1986). The difference in crystal violet intensity observed in uninfected GM-CSF- versus uninfected CSF-1-derived macrophages was due to an inherent inability of GM-CSF-derived cells to incorporate the crystal violet and not due to differences in cell densities. The cell densities of GM-CSF- and CSF-1-derived monolayers were verified

Figure 14. Sensitivity of bone marrow-derived macrophages to infection with vesicular stomatitis virus (VSV). C3H/HeJ bone marrow progenitors were cultured for 7 days in liquid culture in the presence of nCSF-1 or rGM-CSF as described in the Materials and Methods. Following this 7 day culture period, mature macrophages were harvested and re-plated (in the absence of CSF) at a cell concentration of 2×10^5 cells per well in 96-well culture plates. The macrophages were allowed to adhere for 4-6 hours. Following this adherence step, the supernatants were removed and 0.1 ml of VSV suspension added at an M.O.I. of 0.1 or 1.0. Twenty-four hours post-infection, the supernatants were removed and each monolayer was fixed, stained, and examined for virus-induced CPE. These results are from a single experiment representative of 5 - 8 separate experiments.



repeatedly by quantitation of total cell protein by the Lowry method. A comparison of infected to uninfected monolayers for each macrophage population showed that while nCSF-1-derived macrophages exhibited $1 \pm 1\%$ CPE, rGM-CSF-derived macrophages exhibited $71 \pm 7\%$ CPE in cultures infected with an M.O.I. of 0.1 for 8 separate experiments. Differences in VSV sensitivity were also apparent in C3H/OuJ bone marrow-derived macrophages, although the CPE induced in C3H/OuJ macrophages derived under the influence of rGM-CSF was less striking ($34 \pm 11\%$ for $n = 5$ separate experiments). C3H/OuJ macrophages derived under the influence of CSF-1 were refractory to VSV-induced CPE.

To address the mechanism by which VSV induced CPE in rGM-CSF-derived macrophages, we compared VSV replication within these two macrophage populations. Culture supernatants were collected 24 hour post-infection from virus-infected macrophages and were assayed for viral yield by viral plaque formation in L929 fibroblast monolayers. Table 4 shows that no significant difference was observed in the virus yields between rGM-CSF- and nCSF-1-infected macrophages. Since the macrophage cultures were infected at an M.O.I. = 0.1 (i.e., with 2×10^4 VSV plaque forming units (PFU)/well or 2×10^5 PFU/ml), the resultant plaquing data derived from the supernatants of infected cells indicate that no significant viral replication occurred. Therefore, increased viral replication in rGM-CSF-derived macrophages cannot account for the differential CPE observed between the two cell types. To ensure that both cell types were equally capable of being infected with VSV, the macrophages were compared 6 hours post-infection for the presence of intracellular VSV as detected by fluorescent antibody staining. Figures 15A, B, C, and D show phase contrast photographs and the corresponding fluorescent photographs of uninfected (Figure 15A) and VSV-infected (Figure 15C) nCSF-1-derived macrophages versus uninfected (Figure 15B) and VSV-infected (Figure 15D) rGM-CSF-derived macrophages. There was no measurable difference in the ability of rGM-CSF-derived macrophages versus nCSF-1-derived macrophages to be infected with VSV, i.e., uninfected controls showed little non-specific fluorescent staining (Figures 15A and 15C), while both populations exhibited 13 - 16% fluorescent cells in VSV-infected cultures (Figures 15B and 15D). This figure is consistent with an M.O.I. = 0.1. Therefore, the failure of

Table 4Plaque Formation in Virus-Infected GM-CSF and CSF-1-Derived MacrophageCulture Supernatants^a

<u>CELL TYPE</u>	<u>VIRUS YIELD (PFU/ML)^b</u>
GM-CSF-DERIVED	4.2×10^5 [1.1×10^5 - 1.5×10^6]
CSF-1-DERIVED	2.8×10^5 [5.0×10^4 - 1.5×10^6]

^a Two $\times 10^5$ rGM-CSF- and nCSF-1-derived macrophages were cultured in 96-well tissue culture plates as described in Figure 14. Macrophages were incubated for 4 - 6 hours, at which time, the culture supernatants were replaced with 0.1 ml of VSV suspension (M.O.I. = 0.1). Macrophages were infected for 24 hours and the virus supernatants removed and quantified for the number of infectious particles in a plaque forming assay.

^b The data represent the geometric means derived from 7 separate experiments for rGM-CSF-derived macrophages and 4 separate experiments for nCSF-1-derived macrophages. The 95% confidence limits for the treatment groups are indicated in brackets.

Figure 15A. Fluorescent antibody detection of VSV viral proteins in mock-infected
CSF-1-derived macrophages. Two $\times 10^5$ mature (D7) nCSF-1-derived macrophages were cultured in 8-well Lab-Tek chamber slides and were allowed to adhere for 4-6 hours. Following this adherence step, macrophage supernatants were removed and replaced with medium only. Culture supernatants were removed 6 hours later, at which time, monolayers were acetone-fixed for subsequent detection of viral protein production using fluorescent antibody analysis. Figure 15A represents the phase contrast (top panel) and corresponding fluorescent photograph (bottom panel) of uninfected, CSF-1-derived macrophage cultures following staining with anti-VSV antibody and a rhodamine-labeled secondary antibody.

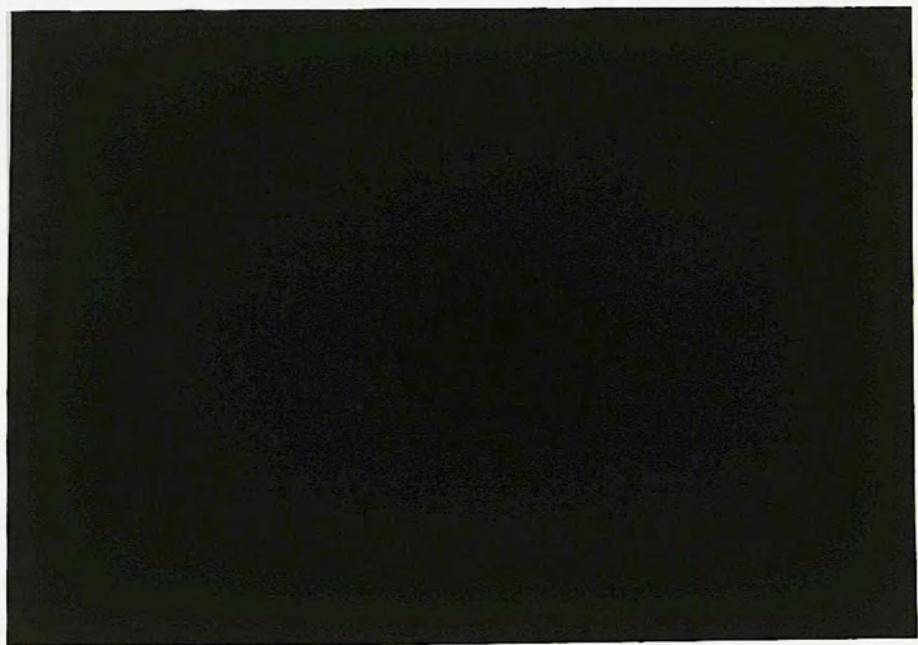
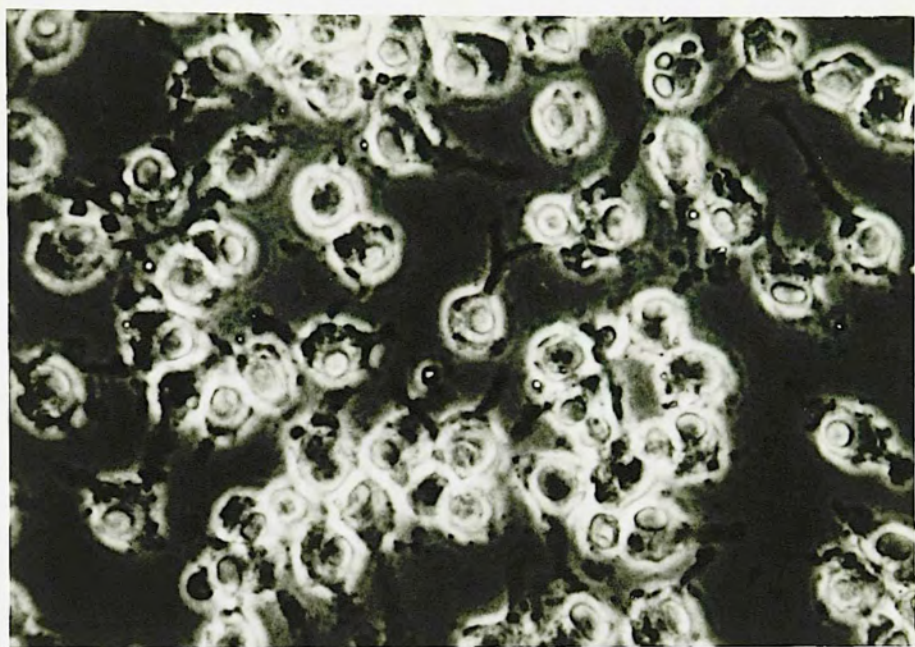


Figure 15B. Fluorescent antibody detection of VSV viral proteins in mock-infected GM-CSF-1-derived macrophages. Two $\times 10^5$ mature (D7) rGM-CSF-derived macrophages were cultured in 8-well Lab-Tek chamber slides and were allowed to adhere for 4-6 hours. Following this adherence step, macrophage supernatants were removed and replaced with medium only. Culture supernatants were removed 6 hours later, at which time, monolayers were acetone-fixed for subsequent detection of viral protein production using fluorescent antibody analysis. Figure 15B represents the phase contrast (top panel) and corresponding fluorescent photograph (bottom panel) of uninfected, rGM-CSF-derived macrophage cultures following staining with anti-VSV antibody and a rhodamine-labeled secondary antibody.

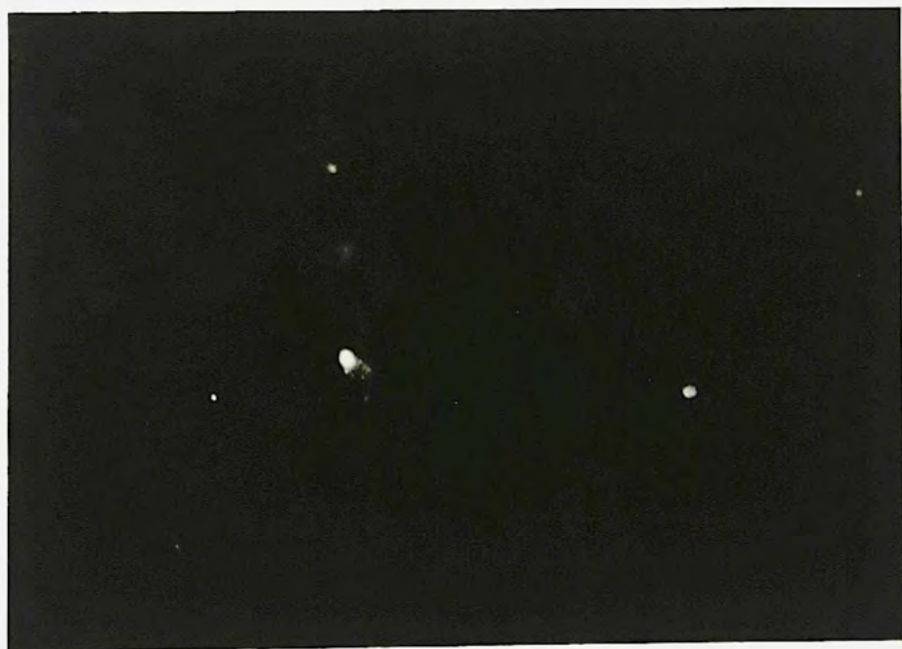
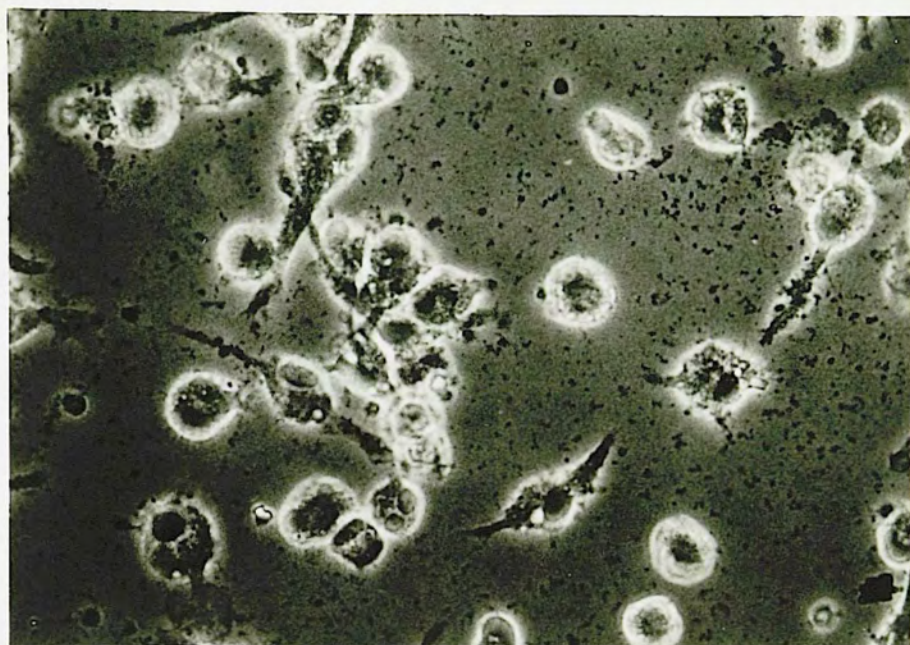


Figure 15C. Fluorescent antibody detection of VSV viral proteins in VSV-infected CSF-1-derived macrophages. Two $\times 10^5$ mature (D7) nCSF-1-derived macrophages were cultured in 8-well Lab-Tek chamber slides and were allowed to adhere for 4-6 hours. Following this adherence step, macrophage supernatants were removed and replaced with 0.1 ml of VSV at an M.O.I. = 0.1. Culture supernatants were removed 6 hours post-infection, at which time, monolayers were acetone-fixed for subsequent detection of viral protein production using fluorescent antibody analysis. Figure 15C represents the phase contrast (top panel) and corresponding fluorescent photograph (bottom panel) of VSV-infected, nCSF-1-derived macrophage cultures following staining with anti-VSV antibody and a rhodamine-labeled secondary antibody.

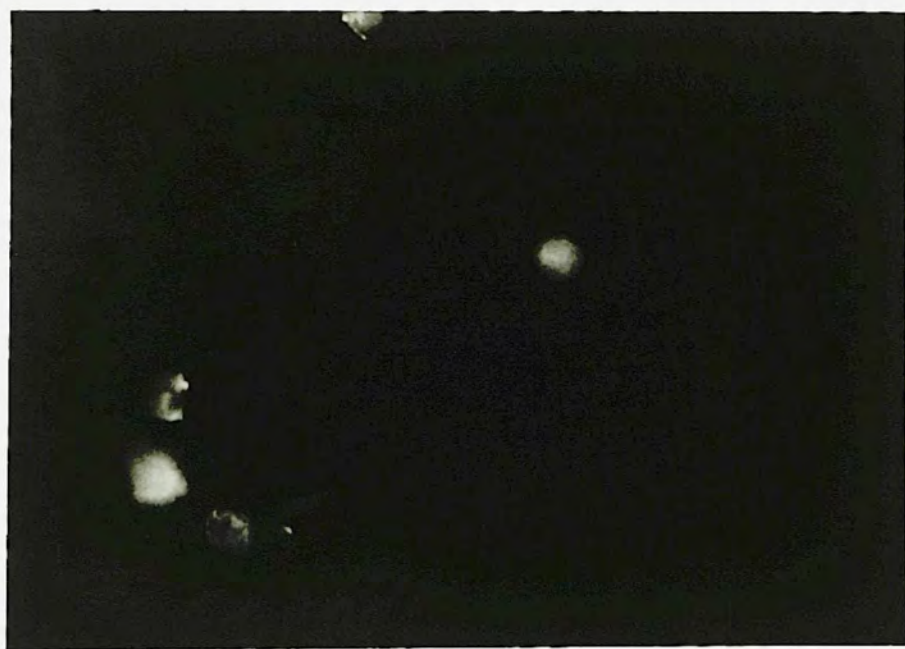
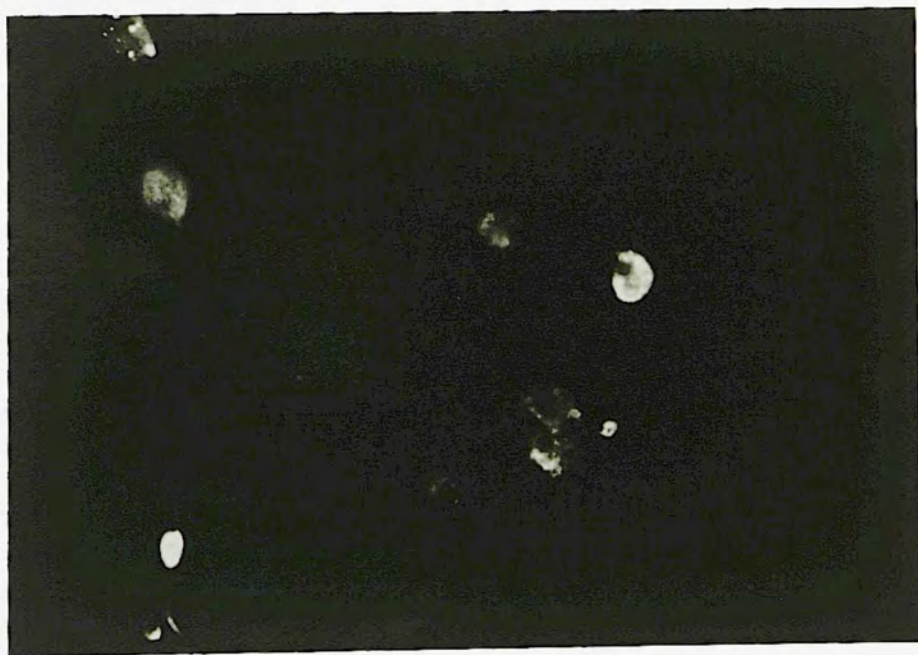
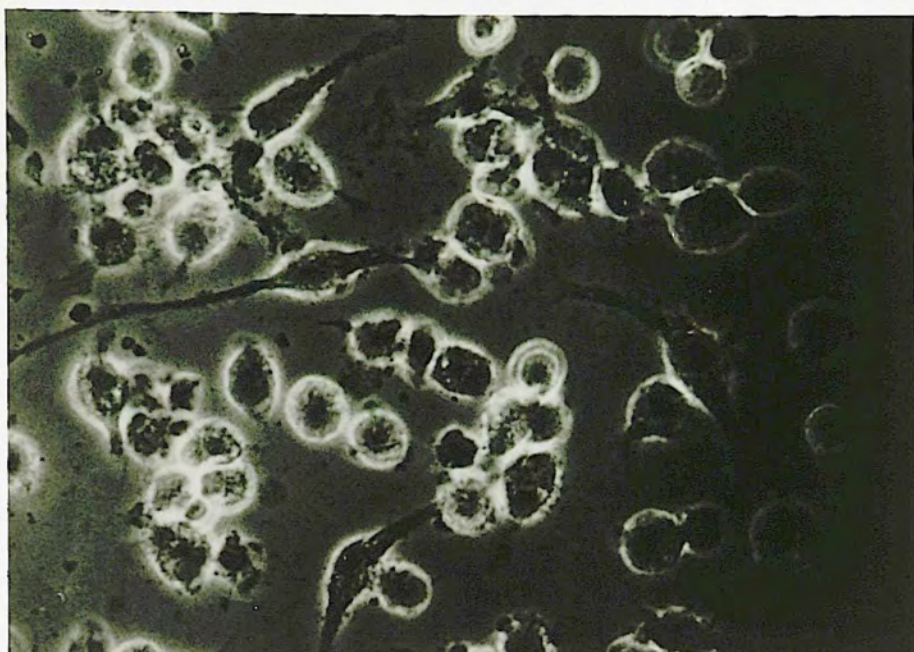


Figure 15D. Fluorescent antibody detection of VSV viral proteins in VSV-infected GM-CSF-derived macrophages. Two $\times 10^5$ mature (D7) rGM-CSF-1-derived macrophages were cultured in 8-well Lab-Tek chamber slides and were allowed to adhere for 4-6 hours. Following this adherence step, macrophage supernatants were removed and replaced with 0.1 ml of VSV at an M.O.I. = 0.1. Culture supernatants were removed 6 hours post-infection, at which time, monolayers were acetone-fixed for subsequent detection of viral protein production using fluorescent antibody analysis. Figure 15D represents the phase contrast (top panel) and corresponding fluorescent photograph (bottom panel) of VSV-infected, rGM-CSF-1-derived macrophage cultures following staining with anti-VSV antibody and a rhodamine-labeled secondary antibody.



VSV-infected CSF-1-derived macrophages to exhibit CPE was not related to an inability of the cells to be infected.

The role of interferon in antiviral protection has been firmly established (reviewed by Gresser, 1984). In addition, the ability of macrophages to produce IFN- α/β has also been well-documented (Ho, 1980; Moore *et al.*, 1984; Warren and Vogel, 1985a; Vogel and Fertsch, 1984). Therefore, the potential role of endogenous IFN- α/β production as a possible mediator of the resistance to VSV CPE in CSF-1-derived macrophages was next examined. Initially, we sought to measure IFN activity in the supernatants of GM-CSF and CSF-1-derived macrophage cultures directly. To do this, the supernatants were tested for their ability to protect L929 fibroblasts in the standard L929 cell antiviral assay. However, the lower limit of sensitivity of the standard L929 fibroblast antiviral assay is approximately 3 U/ml (Vogel *et al.*, 1986). The levels of antiviral activity which were detectable in the GM-CSF-derived supernatants were always below this lower limit, whereas, the levels in CSF-1-derived macrophage supernatants were at the lower limits of detection within the assay. These findings prompted us to seek out a more sensitive method for demonstrating differences in interferon production by GM-CSF- and CSF-1-derived C3H/HeJ macrophages. To increase the sensitivity of detection, serial two-fold dilutions of the NIH Reference Reagent, murine IFN- α/β , were added to the VSV-sensitive, rGM-CSF-derived macrophages to determine the minimal amount of IFN- α/β required to protect these macrophages from VSV-induced CPE. Table 5 demonstrates that the VSV-sensitive, rGM-CSF-derived macrophages were protected from VSV-induced CPE by the addition of as little as 1 U/ml of exogenous IFN- α/β to the cultures 24 hours prior to VSV infection. In contrast, the VSV-resistant, nCSF-1-derived macrophages were treated with serial two-fold dilutions of the NIH Reference Reagent, anti-murine IFN- α/β antibody, in an attempt to demonstrate that the protective factor in CSF-1-derived macrophages was endogenously-produced IFN- α/β . Inclusion of anti-IFN- α/β antibody, but not the control antibody, 24 hours prior to VSV infection of CSF-1-derived macrophages reversed significantly the refractoriness of these cultures to VSV infection (Table 6). CPE was observed in these cultures following treatment with as little as a 1:182 dilution of the NIH Reference Reagent

Table 5

Reversal of Vesicular Stomatitis Virus Sensitivity of GM-CSF-DerivedMacrophages with Exogenous IFN- α/β Treatment

MACROPHAGE <u>CELL TYPE</u>	VSV SENSITIVITY <u>PRIOR TO TREATMENT</u>	TREATMENT PRIOR <u>TO VSV INFECTION^a</u>	VSV SENSITIVITY <u>AFTER TREATMENT</u>
GM-CSF	Sensitive	Medium	Sensitive
GM-CSF	Sensitive	IFN- α/β	Resistant

^a Two $\times 10^5$ C3H/HeJ, rGM-CSF-derived macrophages were re-cultured in the absence of GM-CSF and treated with NIH Reference Reagent IFN- α/β for 24 hours. Following this incubation period, culture supernatants were aspirated and cultures incubated with VSV at an M.O.I. = 0.1 for an additional 24 hours. Cultures were then fixed and stained for assessment of CPE. The amount of IFN- α/β required to protect cells half-maximally was determined spectrophotometrically. The titer required to reverse the observed CPE was 1.0 ± 0.2 U/ml. The data represent the arithmetic mean \pm SEM for n = 6 separate experiments.

Table 6

Reversal of Vesicular Stomatitis Virus Resistance of CSF-1-Derived Macrophages
with Anti-IFN- α/β Treatment

<u>MACROPHAGE</u> <u>CELL TYPE</u>	<u>VSV SENSITIVITY</u> <u>PRIOR TO TREATMENT</u>	<u>TREATMENT PRIOR</u> <u>TO VSV INFECTION^a</u>	<u>VSV SENSITIVITY</u> <u>AFTER TREATMENT</u>
CSF-1	Resistant	Control Antibody	Resistant
CSF-1	Resistant	Anti-IFN- α/β	Sensitive

^a Serial two-fold dilutions of the NIH Reference Reagents anti-IFN- α/β antibody or control antibody (starting dilution=1:10 final concentration) were prepared and used to treat nCSF-1-derived macrophages for 24 hours prior to infection of macrophages with VSV at an M.O.I. = 0.1. The titer of anti-IFN- α/β antibody required to reverse the resistance to VSV infection half-maximally was determined by measuring CPE spectrophotometrically following virus infection and was shown to be 182.4 [93.5 - 355]. The data represent the reciprocal of the dilution required for half-maximal reversal of the VSV resistance and is expressed as the geometric mean of the titer [95% confidence limits] for n = 9 separate experiments.

anti-murine IFN- α/β . However, the control antibody (NIH Reference Reagent No. G-025-501-568) failed to reverse the refractoriness to VSV, even at the highest concentration tested (1:20).

Therefore, the difference observed between nCSF-1- and rGM-CSF-derived macrophages in sensitivity to VSV infection can be related to differences in endogenous IFN- α/β production by the two populations. rGM-CSF-derived macrophages exhibit lower levels of endogenous IFN- α/β in contrast to the levels exhibited by CSF-1-derived macrophages. Lastly, the anti-IFN- α/β antibody failed to reverse the refractoriness of macrophages derived from C3H/OuJ mice under the influence of nCSF-1, even at the highest concentration of antibody tested (a 1:20 dilution of antibody).

The differences observed between GM-CSF- and CSF-1-derived macrophages examined thus far have all been characteristics and/or functions associated with less differentiated macrophages. Therefore, we next examined these two populations with respect to functions associated with more differentiated or activated macrophages. The first of these "higher order" characteristics to be examined was that of Ia antigen expression and one of its associated functions, the ability to stimulate antigen-specific T cell proliferation. Ia antigen expression has been shown to be increased in more differentiated macrophages (Beller *et al.*, 1980) and is necessary for the appropriate presentation of processed antigen to T cells which bear receptors specific for antigen in the context of a specific Ia molecule (Rosenthal and Shevach, 1973; Unanue and Allen, 1987; Heber-Katz *et al.*, 1982). It is this extremely specific interaction which leads to the activation and expansion of the antigen-specific T cell (Rosenthal and Shevach, 1973; Unanue and Allen, 1987) and is critical to the immunocompetence of an animal.

Examination of Ia antigen expression on GM-CSF- and CSF-1-derived macrophages. To

compare the number of Ia-positive cells in macrophage cultures derived from bone marrow cells differentiated in the presence of either rGM-CSF or CSF-1, macrophages were re-cultured in the absence of CSF at precise cell densities, as described in the Materials and Methods. On sequential days over a three day culture period, the percentage of Ia-positive macrophages was determined by antibody and complement-mediated cytotoxicity (Figure 16). In untreated macrophages (i.e., those cultured in the presence of medium alone) there was no significant difference in the percentage of Ia-positive cells in either rGM-CSF- or CSF-1-derived macrophage cultures (approximately 15% - 20%) and this basal level of Ia-positive cells did not change significantly over the three day culture period. When cultures of rGM-CSF- and natural or recombinant CSF-1-derived macrophages were treated on Day 0 with 5.0 U/ml rIFN- γ , both populations of macrophages exhibited an increase in the percentage of Ia-positive cells. This response was most striking after 48 or 72 hours of culture (Days 2 and 3), with CSF-1-derived macrophages exhibiting the greatest increase in the percentage of Ia-positive cells. Specifically, at Day 3, the percentage of Ia-positive cells increased in CSF-1-derived cultures from approximately 15% to 50% with rIFN- γ , whereas the percentage of Ia-positive cells in the rGM-CSF-derived cultures was increased to only 30%. The viability of both cell types, as assessed by trypan blue exclusion, remained high (>95%) over the three day culture period.

Antibody and complement-mediated cytotoxicity measurements only provide information about the percentage of Ia-positive cells, without regard to the total amount of Ia antigen expressed within the population. An ELISA assay was carried out to compare the total Ia expression within these two populations of cells over a three day culture period in the absence and presence of rIFN- γ (Figure 17). In the absence of rIFN- γ , the basal level of Ia expression (medium alone) remained relatively constant over the three day culture period for both CSF-1- and rGM-CSF-derived macrophages. However, rGM-CSF-derived macrophages exhibited a much higher basal level of Ia expression than did CSF-1-derived macrophages. This finding was further substantiated by analysis of steady state, Ia-specific mRNA in medium-treated rGM-CSF- versus CSF-1-derived cultures on Day 0. Cytoplasmic RNA was

Figure 16. Examination of percent Ia positive cells in rGM-CSF- and CSF-1-derived macrophages. Two $\times 10^5$ macrophages were cultured in the absence of colony stimulating factor and were assayed on Days 1, 2, and 3 following treatment of cultures with either medium or 5.0 U/ml rIFN- γ . The percentage of Ia positive cells was determined by antibody and complement-mediated cytotoxicity and uptake of trypan blue. For each individual experiment, 200 cells per culture were scored for viability and duplicate cultures per treatment were assayed. The data represent the arithmetic mean \pm SEM for n = 6 - 7 individual experiments for rGM-CSF and n = 10 - 11 individual experiments for CSF-1 (using either highly purified or HU-CSF-1).

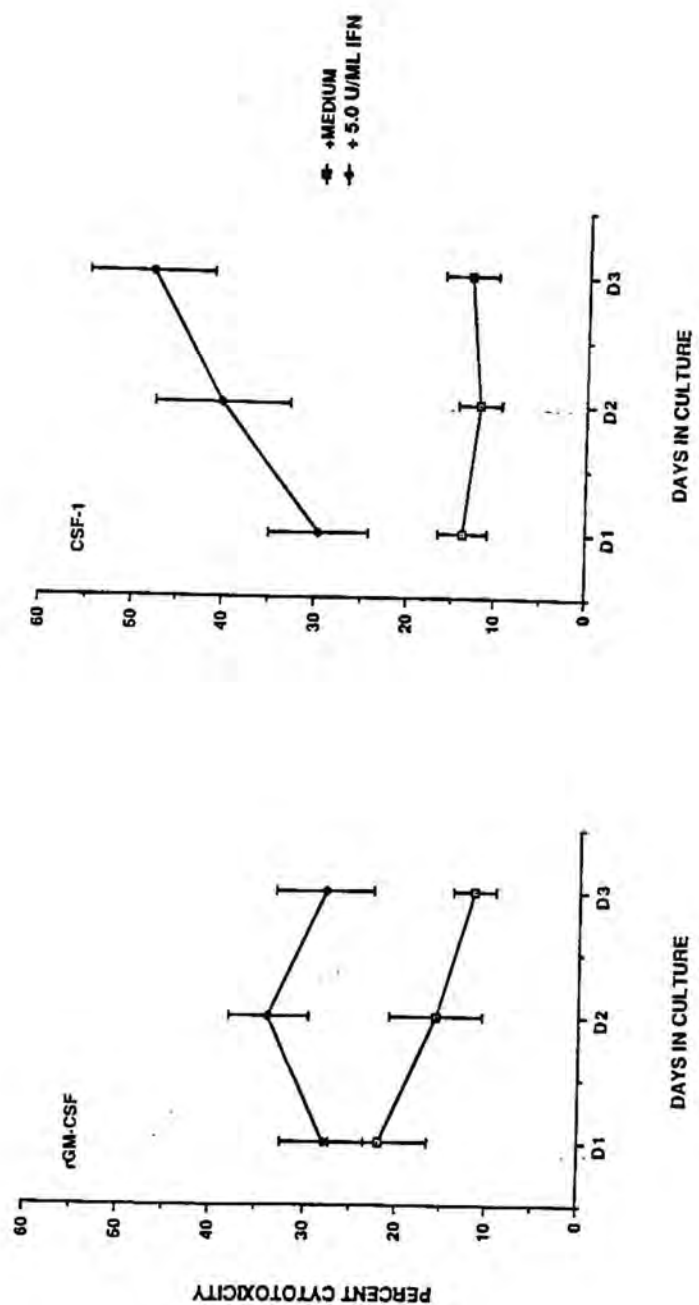
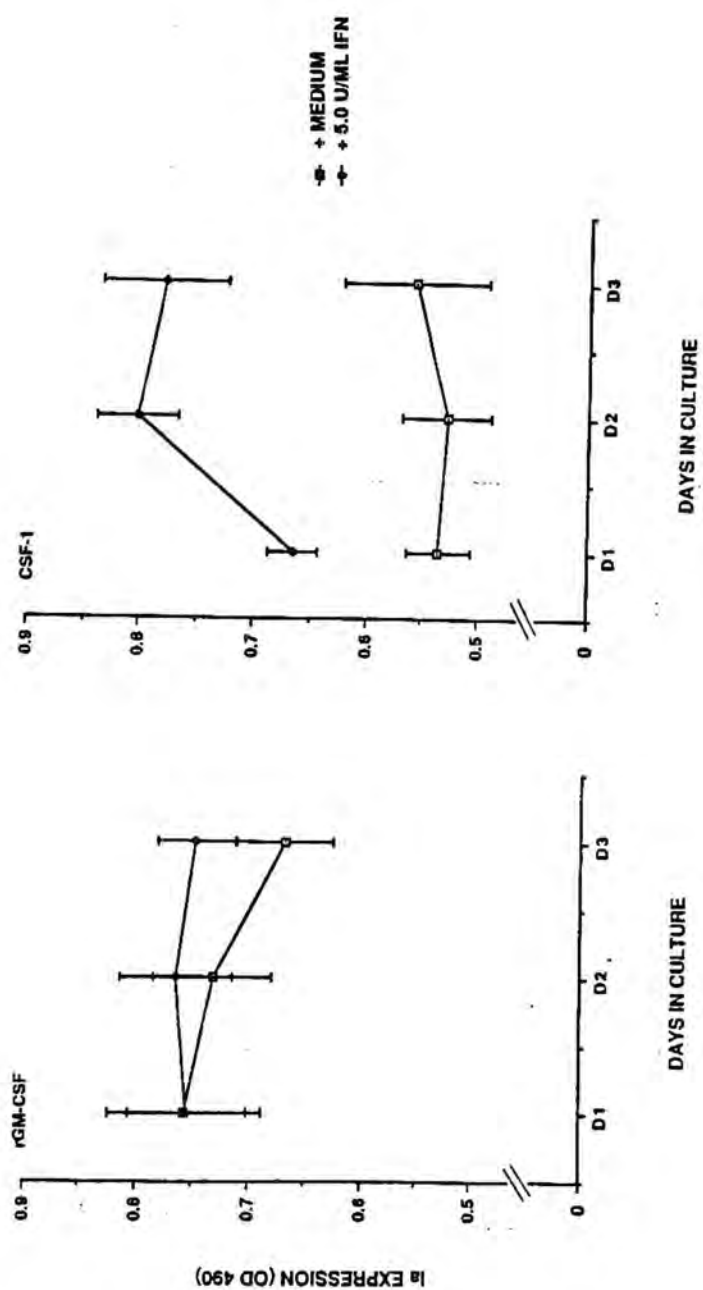


Figure 17. Examination of total Ia expression by ELISA in rGM-CSF- and CSF-1- derived macrophages. Two $\times 10^5$ macrophages were cultured in the absence of CSF and were assayed on D1, D2, and D3 following treatment with either medium or 5.0 U/ml rIFN- γ . The monolayers were paraformaldehyde-fixed and the total Ia expression determined by ELISA. For each individual experiment, 4 replicates per treatment were assayed. The data represent the arithmetic mean \pm SEM for n = 6 -7 individual experiments for rGM-CSF and n = 10 - 13 individual experiments for CSF-1 (using either highly purified or HUrCSF-1).

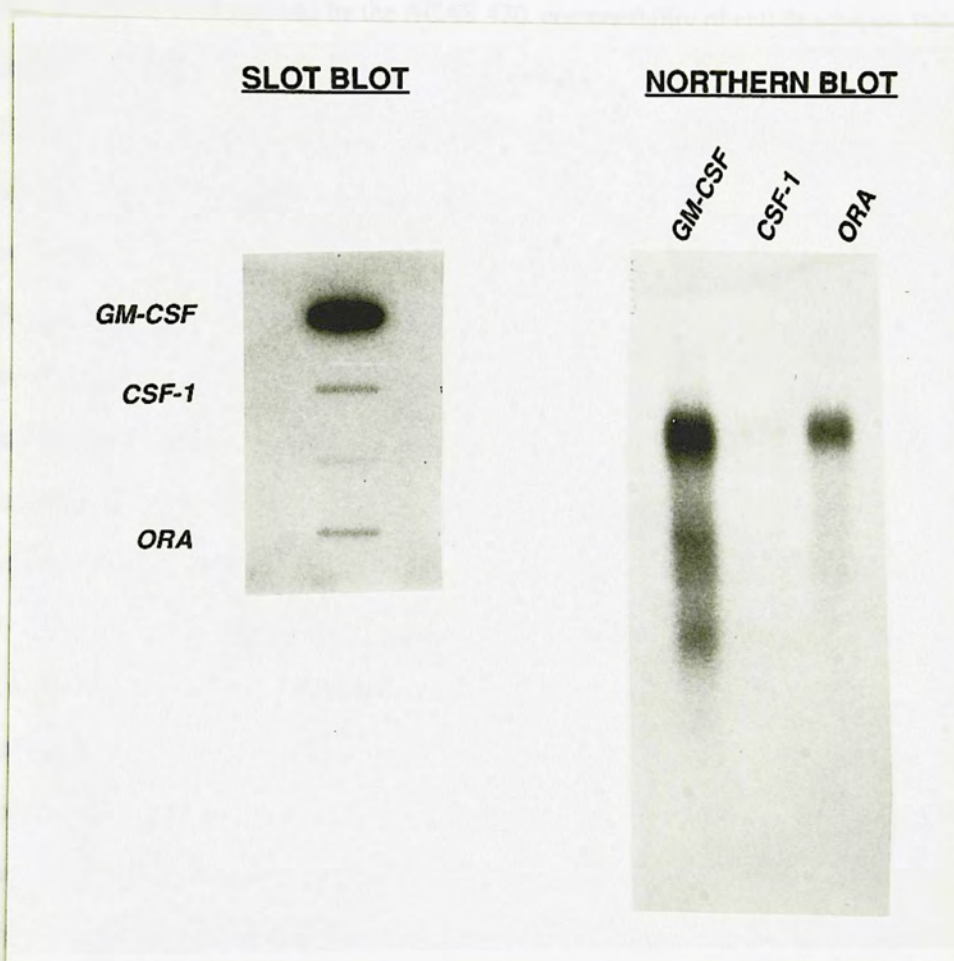


isolated from both populations and was examined by slot blot analysis for quantitation of Ia-specific, steady-state RNA (Figure 18). This finding was also confirmed by Northern blot analysis (Figure 18). Using either method of analysis, it was determined from densitometric scans of autoradiograms that untreated rGM-CSF-derived macrophages possessed approximately four-fold greater quantities of Ia-specific, steady-state RNA than CSF-1-derived macrophages. The difference in Ia-specific mRNA observed on Day 0 was also observed at the level of cell surface Ia antigen expression. Examination of Ia antigen expression by fluorescent activated cell sorting analysis showed that GM-CSF-derived macrophages exhibited greater levels of cell surface Ia antigen expression than CSF-1-derived macrophages (data not shown).

Treatment of rGM-CSF-derived macrophages with rIFN- γ did not increase their level of total Ia expression as measured by ELISA (Figure 17), i.e., expression of total Ia remained at the already elevated levels observed in medium-treated cultures. However, in the presence of 5.0 U/ml rIFN- γ , CSF-1-derived macrophages increased their total Ia expression to levels exhibited by rGM-CSF-derived cultures.

Determination of Ia density by fluorescent antibody analysis. Since the numbers of Ia-positive cells were approximately equivalent in cultures of medium-treated rGM-CSF- and nCSF-1-derived macrophages (Figure 16), but the total Ia expression, as measured by ELISA, was significantly greater in rGM-CSF-derived cells (Figure 17), this led to the hypothesis that the density of Ia on rGM-CSF-derived macrophages was significantly greater than on nCSF-1-derived cells. To test this hypothesis, the density of Ia on individual macrophages was examined through the use of the ACAS 470 which allows for quantitative analysis of fluorescent cell surface markers on individual cells within an adherent cell population. The use of the ACAS 470 has eliminated the concern of potential damage to adherent cells which must be detached mechanically or enzymatically for analysis of cell surface markers by standard flow cytometry. Therefore, the ACAS 470 provides more flexibility and reduces the potential for artifacts since the cells treated in culture can be examined without having to be

Figure 18. Analysis of Ia-specific mRNA derived from rGM-CSF- and nCSF-1-derived macrophages. The autoradiograms from a slot blot (in which 10 µg RNA per slot was applied to the nitrocellulose paper) and a Northern blot (in which 5 µg RNA per lane was applied to the gel) are shown following hybridization to a ^{32}P -cDNA Ia-specific probe. The autoradiogram shown is representative of 4 separate experiments. The ORA cell line was used as a source of constitutively produced, Ia-specific mRNA.



detached for analysis. In addition, the degree of fluorescence associated with a particular cell can be visualized through the use of a pseudo-color scale (with white being the highest level of intensity).

Figures 19A and B illustrate a representative experiment utilizing the ACAS 470 to detect cell surface Ia antigen expression in macrophage cultures from rGM-CSF- or CSF-1-derived cultures. For each field scanned by the ACAS 470, comparability of cell density per field was verified by phase contrast microscopy. In this experiment, each field contained between 80 and 110 cells, as assessed by counting the number of cells in the individual phase contrast photographs. Figures 19A and B illustrate the distribution of fluorescent staining for Ia expression in medium-treated (Figure 19A) and rIFN- γ -treated (Figure 19B) rGM-CSF- and nCSF-1-derived macrophages. Figure 19A demonstrates that although the number of Ia-positive cells were found to be equivalent in the two macrophage populations by antibody and complement-mediated cytotoxicity (Figure 16), the density of Ia expression on medium-treated, nCSF-1-derived macrophages (Figure 19A) was so low when compared to the fluorescence exhibited by rGM-CSF-derived macrophages (Figure 19A), that most of the nCSF-1-derived cells could not be visualized. However, following rIFN- γ treatment (Figure 19B), there was an increase in the number of fluorescent cells within the nCSF-1-derived population, as well as a marked increase in the intensity of fluorescence. However, the density of Ia per nCSF-1-derived macrophage following IFN- γ treatment was still less than that seen in medium- or rIFN- γ -treated rGM-CSF-derived cells. Treatment of rGM-CSF-derived cultures with rIFN- γ failed to alter significantly the number of positive cells detected by the ACAS 470 or the already high density of Ia per cell (Figure 19B).

Measurement of T cell proliferation induced by rGM-CSF- and CSF-1-derived macrophages. The relationship of Ia antigen expression to the ability of macrophages to function as antigen-presenting cells has been well-documented (Unanue and Allen, 1987; Beller, 1984; Lee and Wong, 1980; and Unanue *et al.*, 1984). Since differences in Ia expression were observed in these two cell populations, the capacity of these cultures to induce T cell

Figure 19A. Ia density analysis by fluorescence in medium-treated CSF-1- and

rGM-CSF-derived macrophages. Macrophages (1×10^5 /culture) were treated with medium for 2 to 3 days and were analyzed for Ia antigen expression using the ACAS 470. To control for background fluorescence, cells treated with fluorescenated secondary antibody only were also tested and showed little detectable fluorescence. The fields scanned on these control cultures were established as the baseline fluorescent threshold and were subtracted from all of the other scans to eliminate this low background of non-specific fluorescence. Figure 19A represents fluorescent photographs of (A) CSF-1-derived macrophages and (B) rGM-CSF-derived macrophages. Each figure represents a single field representative of 4 individual fields which were verified by phase contrast microscopy to contain approximately the same number of cells (i.e., 80-110 cells per field).

A.



B.

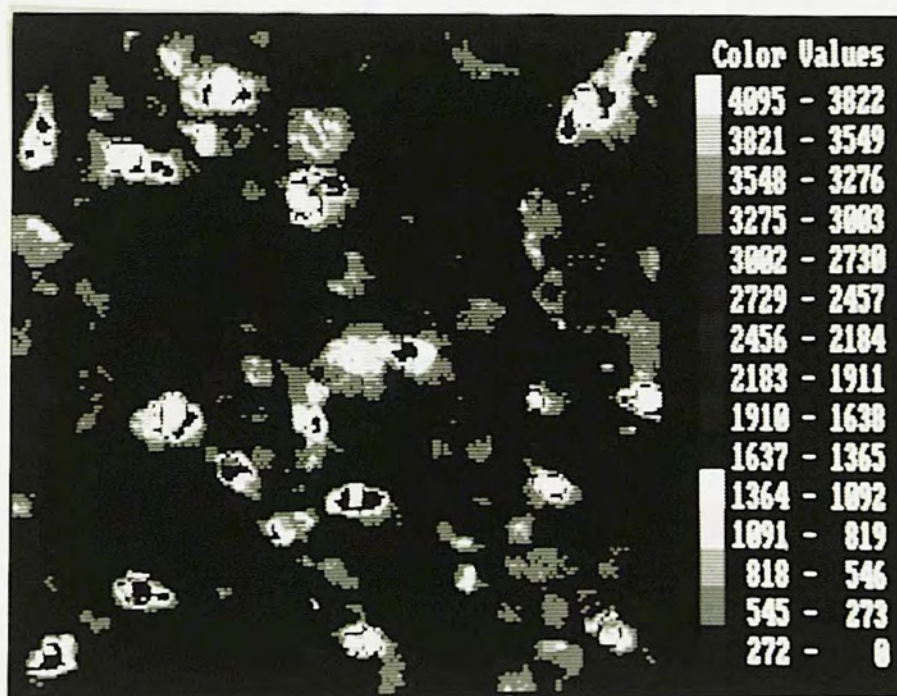
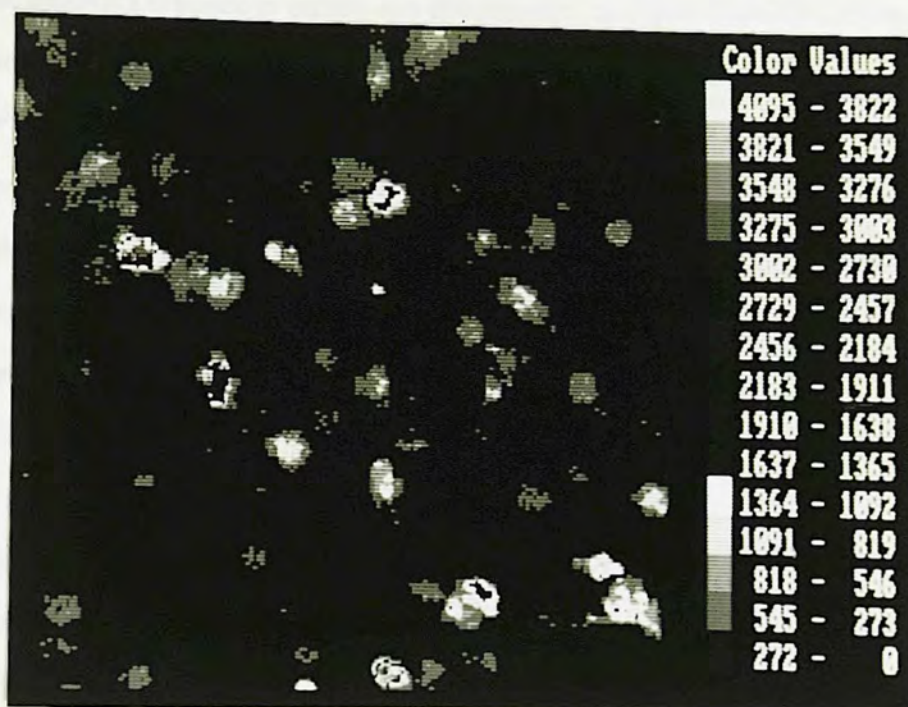
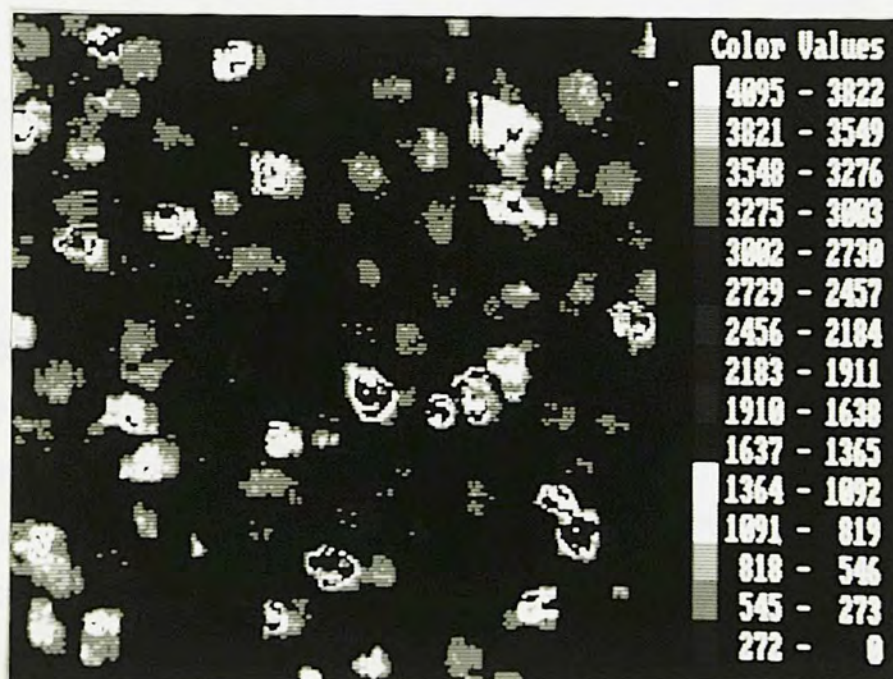


Figure 19B. Ia density analysis by fluorescence in rIFN- γ -treated CSF-1- and rGM-CSF-derived macrophages. Macrophages (1×10^5 /culture) were treated with 5.0 U/ml of rIFN- γ for 2 to 3 days and were analyzed for Ia antigen expression using the ACAS 470. To control for background fluorescence, cells treated with fluorescenated secondary antibody only were also tested and showed little detectable fluorescence. The fields scanned on these control cultures were established as the baseline fluorescent threshold and were subtracted from all of the other scans to eliminate this low background of non-specific fluorescence. Figure 19B represents fluorescent photographs of (A) CSF-1-derived macrophages and (B) rGM-CSF-derived macrophages. Each figure represents a single field representative of 4 individual fields which were verified by phase contrast microscopy to contain approximately the same number of cells (i.e., 80-110 cells per field).

A.



B.

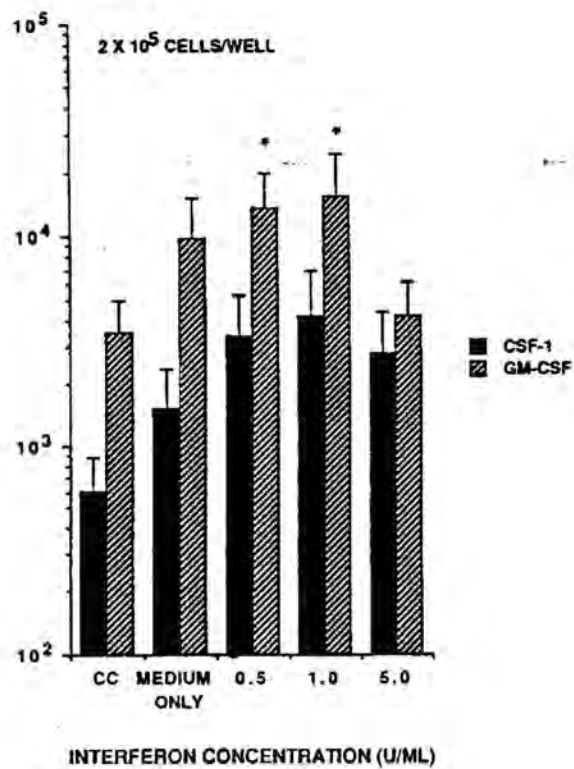
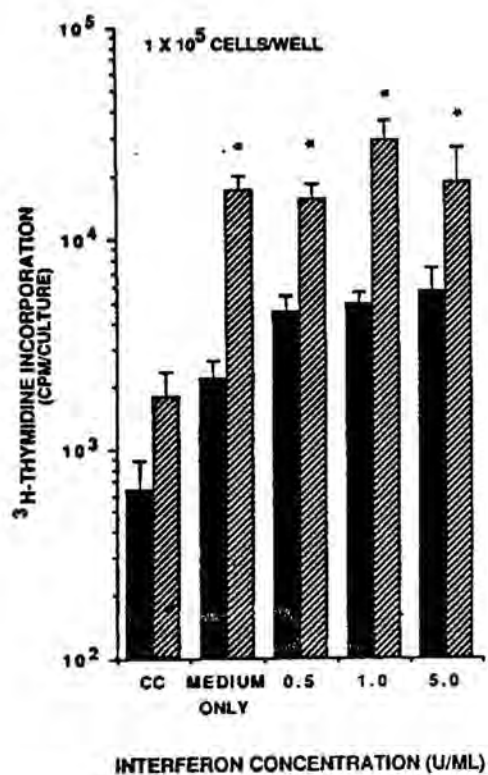


proliferation following exposure to antigen was also assessed. In Figure 20, rGM-CSF- or nCSF-1-derived macrophages were cultured at two different cell concentrations (i.e., 1×10^5 and 2×10^5 cells/culture) and were incubated with medium or rIFN- γ (0.5, 1.0, or 5.0 U/ml) for two or three days. At that time, cells were antigen-pulsed with KLH and irradiated. The ability of T cells derived from KLH-primed mice to proliferate in response to antigen when presented by these macrophages was measured by uptake of tritiated thymidine. T cells alone or macrophages alone consistently incorporated less than 500 cpm per culture. In the cell controls [CC; macrophages plus T cells (not antigen-pulsed)], rGM-CSF-derived macrophages were more stimulatory for T cells than nCSF-1-derived macrophages. When medium-treated macrophages were pulsed with KLH and then co-cultured with antigen-primed T cells, rGM-CSF-derived macrophages were capable of stimulating greater T cell proliferation than medium-treated nCSF-1-derived cells. Recombinant IFN- γ treatment had little effect on the ability of rGM-CSF-derived macrophages to stimulate T cell proliferation; however, treatment of nCSF-1-derived macrophages with IFN- γ (prior to pulsing with KLH) increased their ability to stimulate T cell proliferation slightly, although this difference was not found to be statistically significant. At 2×10^5 macrophages/well, a similar pattern of T cell proliferation was observed; however, at the highest concentration of rIFN- γ used (5.0 U/ml), a depression of T cell proliferation induced by the antigen-pulsed rGM-CSF-derived macrophages was observed.

Basal expression of PGE production by GM-CSF- and CSF-1-derived macrophages.

Prostaglandins have been shown by a number of investigators to inhibit Ia expression in both resident and elicited peritoneal macrophages (Tripp *et al.*, 1986; Snyder *et al.*, 1982). Prostaglandins have also been shown to inhibit mitogen-induced T lymphocyte proliferation (Kuehl, 1977; Hoffeld *et al.*, 1980; Gernsma, 1982; Morley, 1982). Since nCSF-1-derived macrophages exhibited lower basal levels of Ia expression than rGM-CSF-derived macrophages (Figures 17 and 19B), as well as a lesser ability to induce proliferation of T lymphocytes in the absence or presence of antigen (Figure 20), we examined the levels of PGE produced in

Figure 20. Comparison of T cell proliferation induced by rGM-CSF- and nCSF-1-derived macrophages. Macrophages were cultured at 1×10^5 /culture (Fig. 23A) or 2×10^5 /culture (Fig. 23B) in the absence or presence of rIFN- γ for 2 - 3 days. At that time they were pulsed with antigen (KLH) for 4 hours, irradiated, and co-cultured with KLH-primed, nylon wool-purified, lymph node T cells for an additional 4 days. Tritiated thymidine was added to cultures on Day 4 and the radioactive incorporation measured by liquid scintillation counting. The data represent the arithmetic mean \pm SEM for 4 individual experiments. The cell control (CC) values (cpm incorporated into macrophages and T cells in the absence of antigen) have not been subtracted from the experimental values. Within certain treatment groups (those indicated by an asterisk), rGM-CSF-derived macrophages induced significantly greater antigen-specific T cell proliferation ($p \leq 0.05$) than CSF-1-derived macrophages.



rGM-CSF- vs. nCSF-1-derived cultures (Table 7). Macrophages derived from bone marrow progenitors in the presence of rGM-CSF produced approximately 10-fold higher levels of PGE than macrophages derived in the presence of nCSF-1. Therefore, it is unlikely that the reduced levels of Ia expression and induction of antigen-specific T cell proliferation exhibited by nCSF-1-derived macrophages could result from the presence of high PGE production in these cultures.

Examination of the development of tumoricidal activity in GM-CSF- and CSF-1-derived macrophages. Fully activated macrophages have been shown to acquire the capacity to kill tumors in vitro (Cohn, 1978). To extend the functional characterization of GM-CSF- and CSF-1-derived macrophages, we examined the basal tumoricidal activity, as well as the signal requirements of these two populations for activation to a fully tumoricidal state. Historically, Hibbs et al. (1977) demonstrated that activation of macrophages to a fully tumoricidal state was a multi-signal event: macrophages were first "primed" into a stimulated, non-cytolytic state by a lymphokine referred to as "Macrophage Activating Factor" or "MAF". Once "primed", the macrophage could be activated to full tumoricidal capacity by a "trigger" signal, such as LPS. Additional studies demonstrated that IFN- γ could serve as a potent source of MAF (Pace et al., 1983a, 1983b; Schreiber et al., 1983). Protein-free, phenol-water-extracted LPS can serve as a "trigger" signal for LPS-responsive (Lps^N) macrophages, but not for the C3H/HeJ (Lps^d) macrophages (Pace and Russell, 1981; Pace et al., 1985a). However, if one uses as a trigger signal butanol-extracted LPS (But-LPS), which contains significant levels of lipid A-associated protein, IFN- γ -primed macrophages from either Lps^N and Lps^d mice can recognize these molecules as a trigger signal and be activated to a fully tumoricidal state (Hogan and Vogel, 1987). In the following studies, C3H/HeJ macrophages derived from BMP under the influence of rGM-CSF or nCSF-1 were examined for their signal requirements for activation to a fully tumoricidal state.

Table 7Prostaglandin Production by GM-CSF and CSF-1-Derived Macrophages^a

MACROPHAGE	
<u>CELL TYPE</u>	<u>PROSTAGLANDIN E PRODUCTION (ng/ml)^b</u>
rGM-CSF-derived	11.5 [3.7 - 19.3]
nCSF-1-derived	1.5 [0.3 - 2.7]

^a Supernatants from medium-treated cultures were harvested at either 24 or 48 hours and assayed for prostaglandin activity by radioimmunoassay as described in the Materials and Methods.

^b The data represents the arithmetic mean [95% confidence limits] derived from n = 9 separate experiments for rGM-CSF and n = 8 separate experiments for nCSF-1.

Comparison of tumoricidal activity of GM-CSF- and CSF-1-derived macrophages.

In this series of experiments, the signal requirements for activation of macrophages derived from bone marrow progenitors under the influence of either rGM-CSF or nCSF-1 to kill tumor cells were compared. The dose range of priming and triggering signals used was based on previous studies using thioglycollate-elicited peritoneal exudate macrophages (Hogan and Vogel, 1987). In response to medium alone (i.e., no rIFN- γ as a "priming" signal and no But-LPS as a "triggering" signal), rGM-CSF-derived macrophages exhibited a significantly higher basal level of tumoricidal activity than nCSF-1-derived macrophages (Figure 21; $16 \pm 5\%$ versus $6 \pm 3\%$ cytotoxicity).

In the presence of a "priming" signal only (i.e., rIFN- γ), both rGM-CSF- and nCSF-1-derived macrophages were stimulated approximately two-fold above basal levels of tumor cytotoxicity (Figure 21); however, the increase observed in the nCSF-1-derived macrophage cultures was not statistically significant. At both concentrations of rIFN- γ tested, rGM-CSF-derived macrophages maintained a significantly greater capacity to kill tumor cells than nCSF-1-derived macrophages.

In response to the "triggering" signal only (i.e., But-LPS), there was no change from the basal levels in the capacity of rGM-CSF-derived macrophages to kill tumor cells *in vitro* (Figure 22). However, at the highest concentration of But-LPS tested (5.0 $\mu\text{g/ml}$), the tumoricidal capacity of nCSF-1-derived macrophages was increased to the same level exhibited by medium- or But-LPS-treated rGM-CSF-derived macrophages. Increasing the concentrations of But-LPS to as much as 20 $\mu\text{g/ml}$ led to no significant increase in the tumoricidal activity above that seen with 5 $\mu\text{g/ml}$.

Figure 23 presents a comparison of the responses of rGM-CSF- and nCSF-1-derived macrophages to medium alone, rIFN- γ alone, But-LPS alone, and finally, to the two signals in combination. In rGM-CSF-derived cultures (left panel), no increase in tumoricidal activity was induced by combined treatment with a low concentration of rIFN- γ (1.0 U/ml) and any

Figure 21. Response of bone marrow-derived macrophages to a "priming" signal (rIFN- γ) only for generation of increased tumor cytotoxicity. Two $\times 10^5$ rGM-CSF- or nCSF-1-derived macrophages were cultured in the presence of either medium or the indicated concentrations of rIFN- γ for 24 hours prior to the addition of ^{51}Cr -labeled P815 tumor cell targets. Following 16 hours co-culture of macrophages and target cells, chromium release was measured and the percent cytotoxicity determined as described in the Materials and Methods. The data represent the arithmetic mean \pm SEM for $n = 8 - 13$ experiments for rGM-CSF-derived macrophages and $5 - 11$ experiments for CSF-1-derived macrophages. Statistical differences between the two cell types within a given treatment group was determined using a two-way ANOVA as described in the Materials and Methods ($p \leq 0.05$ was chosen as the level of statistical significance). Asterisks indicate statistical differences between rGM-CSF- and CSF-1-derived cultures within a given treatment.

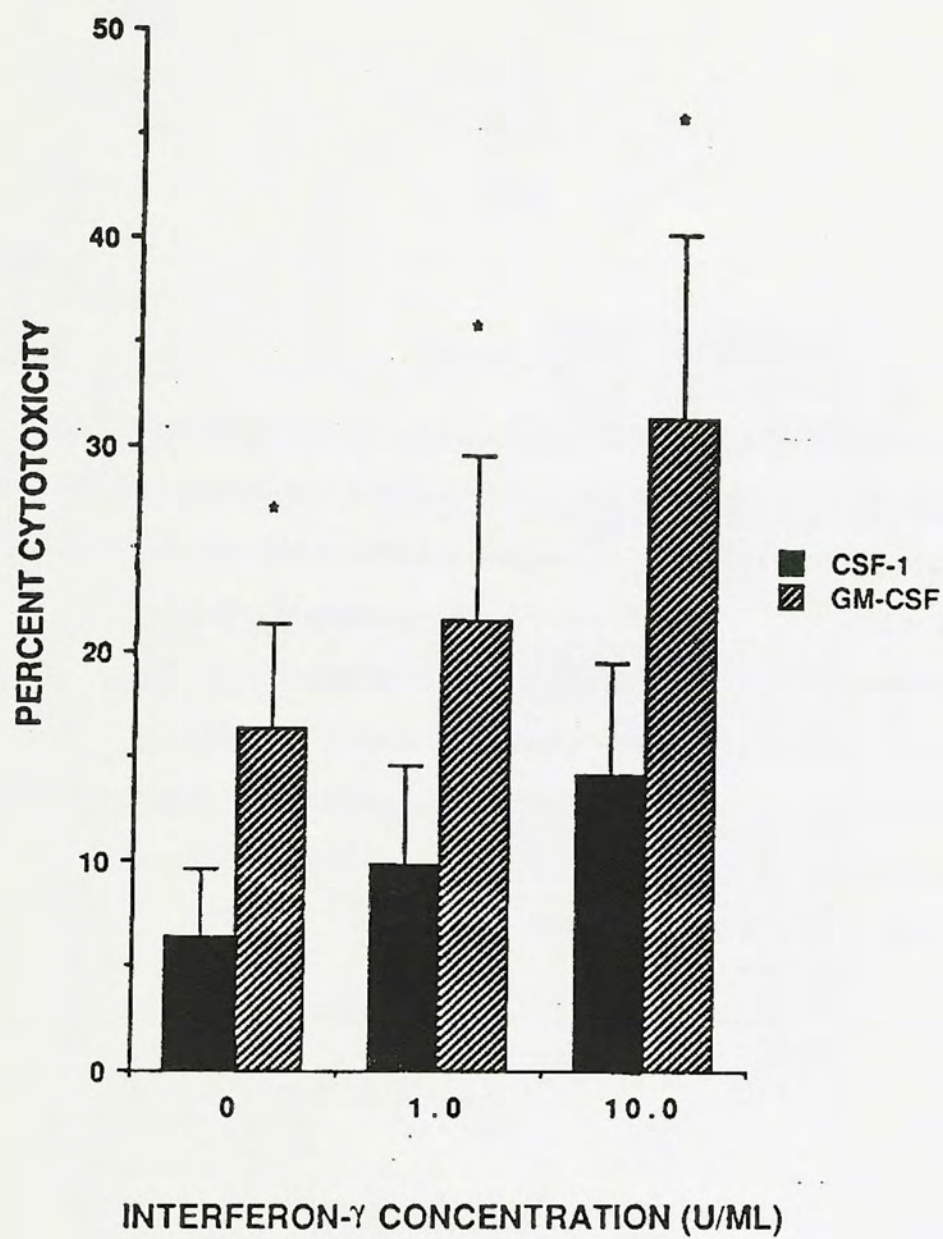


Figure 22. Response of bone marrow-derived macrophages to a "trigger" signal (But-LPS) only for the generation of increased tumor cytotoxicity. Two $\times 10^5$ rGM-CSF- or nCSF-1-derived macrophages were cultured in the presence of medium or But-LPS for 24 hours prior to the addition of ^{51}Cr -labeled P815 tumor cell targets as described in Figure 21. The data represent the arithmetic means \pm SEM for 8 - 13 experiments for rGM-CSF-derived macrophages and 6 - 13 experiments for CSF-1-derived macrophages. Statistical differences between the two cell types within a given treatment group was determined using a two-way ANOVA ($p \leq 0.05$ was chosen as the level of statistical significance). Asterisks indicate statistical differences between rGM-CSF- and CSF-1-derived cultures within a given treatment.

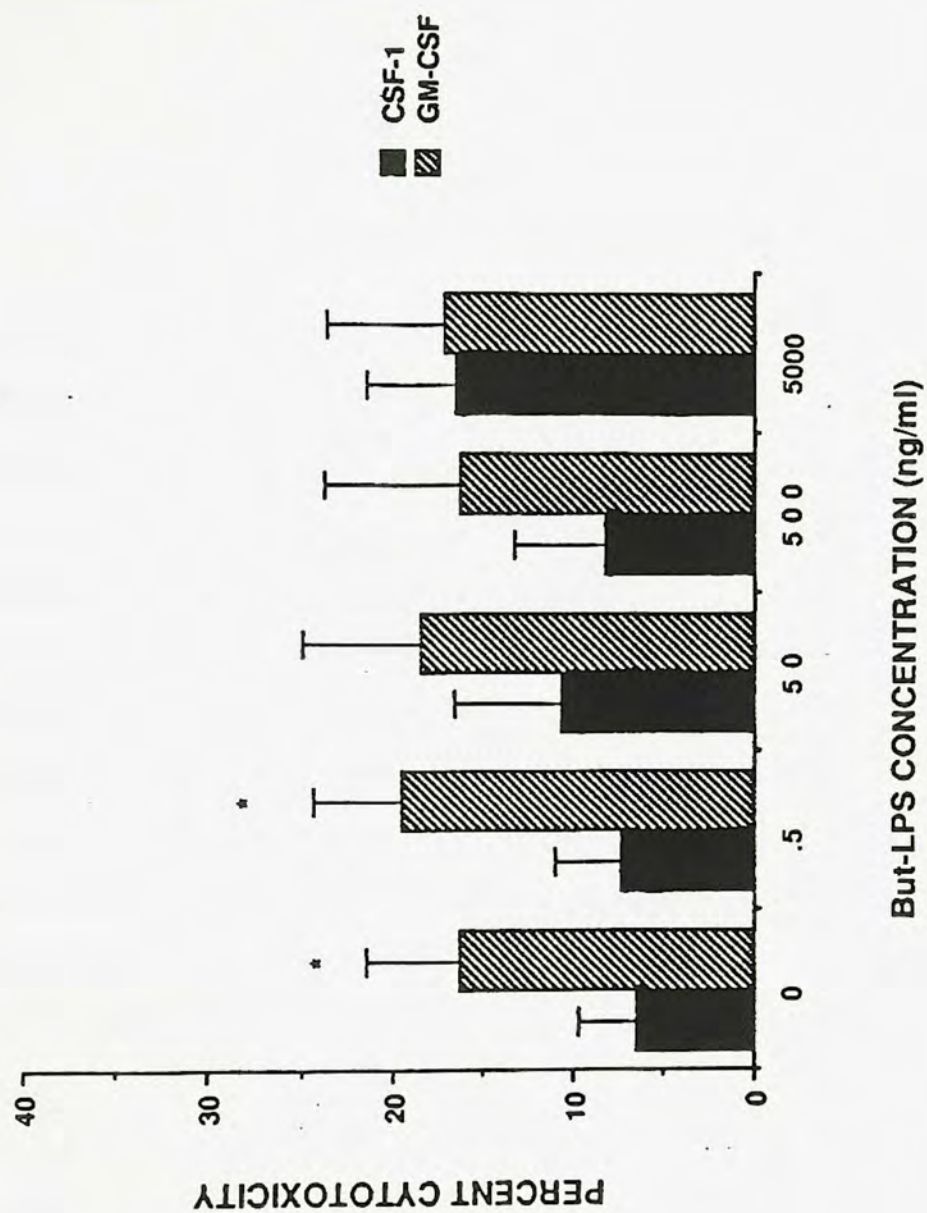
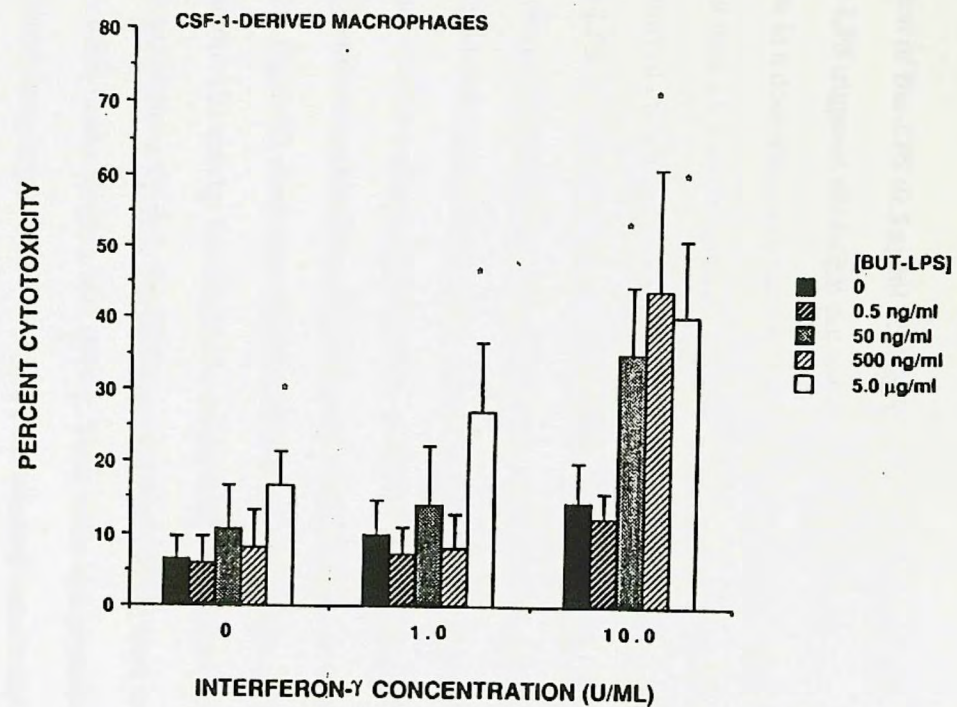
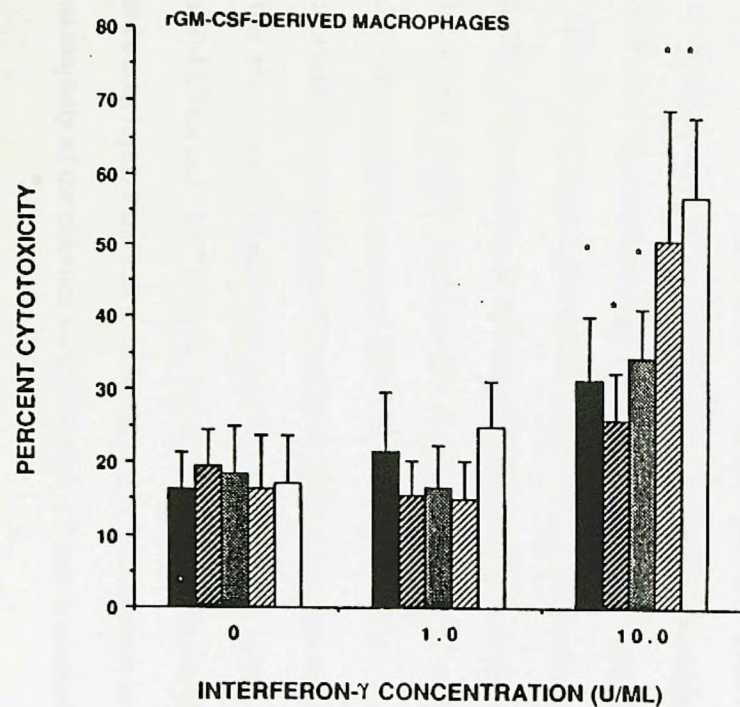


Figure 23. Response of bone marrow-derived macrophages following treatment with rIFN- γ and/or But-LPS for the generation of increased tumor cytotoxicity. Two $\times 10^5$ rGM-CSF- (left panel) or nCSF-1-derived (right panel) macrophages were cultured in the presence of medium alone or the indicated concentrations of rIFN- γ and/or But-LPS for 24 hours prior to the addition of ^{51}Cr -labeled P815 tumor cell targets. Following 16 hr co-culture of macrophages and target cells, chromium release was measured and the percent cytotoxicity determined as described in the Materials and Methods. The data represent the arithmetic mean \pm SEM for $n = 8 - 13$ separate experiments for rGM-CSF-derived macrophages and $n = 5 - 11$ separate experiments for CSF-1-derived macrophages. Statistical differences between the means (medium only versus a specific treatment) were determined using a two-way ANOVA ($p \leq 0.05$ was chosen as the level of statistical significance). Asterisks indicate those treatment groups found to be statistically different from the response of medium-treated cultures.



concentration of But-LPS (0.5 ng/ml - 5.0 µg/ml). However, at the highest dose of rIFN-γ (10.0 U/ml), But-LPS triggered rGM-CSF-derived macrophages to kill tumor cells from approximately 31% to 57% in a dose-dependent fashion. When nCSF-1-derived macrophages (right panel) were treated with 1.0 U/ml rIFN-γ and 5.0 µg/ml But-LPS, levels of cytotoxicity were increased to levels observed in similarly-treated rGM-CSF-derived cultures. In the presence of 10.0 U/ml rIFN-γ, But-LPS increased significantly the cytotoxicity exhibited by these cells over a more limited concentration range (50.0 ng/ml-5.0 µg/ml). The maximal lytic ability of nCSF-1-derived macrophages induced by the two signals never achieved the levels observed in rGM-CSF-derived macrophage cultures in any of eleven individual experiments.

Higher dose combinations of priming and triggering signals were not employed for two reasons. First, Figure 23 shows that for both nCSF-1- and rGM-CSF-derived macrophages, a plateau of tumoricidal activity was obtained over the dose combination range tested. Secondly, Table 8 shows that above 5 µg/ml, But-LPS becomes significantly more toxic to the macrophages when in combination with rIFN-γ. Thus, while both populations of bone marrow-derived macrophages require two signals for maximal tumoricidal activity, rGM-CSF-derived macrophages exhibited significantly higher tumoricidal activity in the presence of medium alone or at the highest concentrations of rIFN-γ and But-LPS.

The preceding experiments were designed to examine the development and differentiation state of mature macrophages following bone marrow progenitor growth in either GM-CSF or CSF-1. The next series of experiments was carried out in the presence of both GM-CSF and CSF-1. The number of colonies which develop in cultures following simultaneous addition of GM-CSF and CSF-1 might provide insights as to whether the differences observed in Figures 3 and 4 were due to the action of GM-CSF on a subset of CSF-1-responsive progenitors or on a unique population of progenitors. Therefore, initial experiments were performed in the soft agar overlay assay, as described in the Materials and Methods, for the development of colonies from GM-CFCs and M-CFCs; however, in these experiments both GM-CSF and CSF-1 were included in the bottom layer of the soft agar overlay assay. In response to either GM-CSF or CSF-1, the vast majority of the colonies which form are < 2 mm in diameter. Under culture

Table 8

Viability of Macrophages Following Treatment with High Doses of But-LPS and/or rIFN- γ ^a

<u>CELL TYPE</u>	<u>MEDIUM^b</u>	<u>rIFN-γ (10.0 U/ml)</u>
<u>GM-CSF-DERIVED:</u>		
Medium	7.6 \pm 1.3	11.5 \pm 3.8
5 μ g/ml But-LPS	5.3 \pm 1.6	8.6 \pm 3.0
10 μ g/ml But-LPS	5.5 \pm 3.3	19.5 \pm 1.9*
15 μ g/ml But-LPS	9.3 \pm 2.3	19.3 \pm 2.4*
20 μ g/ml But-LPS	4.6 \pm 1.0	20.7 \pm 3.3*
<u>CSF-1-DERIVED:</u>		
Medium	3.3 \pm 1.0	4.5 \pm 1.2
5 μ g/ml But-LPS	2.5 \pm 1.3	9.7 \pm 3.1
10 μ g/ml But-LPS	2.6 \pm 1.0	16.0 \pm 3.9*
15 μ g/ml But-LPS	2.2 \pm 0.5	12.4 \pm 2.5
20 μ g/ml But-LPS	3.5 \pm 1.3	15.6 \pm 4.2*

^a Two $\times 10^5$ GM-CSF- or CSF-1-derived macrophages were treated for 24 hours with medium, rIFN- γ (10.0 U/ml) and/or various concentrations of But-LPS. Following the 24-hour incubation period, supernatants were removed and the viability of macrophages determined by trypan blue exclusion.

^b The values are reported as percent cytotoxicity and the data represent the arithmetic mean \pm SEM for 4 separate experiments. The asterick (*) indicate values which are statistically different from medium alone as determined by Student's t-test ($p \leq 0.05$).

conditions of simultaneous GM-CSF and CSF-1 addition, we observed the development of a significant proportion of very large (≥ 2 mm) colonies, indicative of those derived from primitive progenitors with high proliferative potential (Baines *et al.*, 1981; Hagan *et al.*, 1985; Madonna and Vogel, 1985; Schwartz *et al.*, 1986). Additional experiments were performed to characterize this progenitor development.

Stimulation of HPP-CFC progenitors in the combined presence of GM-CSF and CSF-1. The preliminary observation, that combined treatment of bone marrow progenitors with rGM-CSF and nCSF-1 (1000 units of each CSF per culture) resulted in colonies of ≥ 2 mm diameter, prompted us to examine further the formation of these colonies under a variety of conditions. A number of previous investigators (Baines *et al.*, 1981; Hagan *et al.*, 1985; Madonna and Vogel, 1985; Schwartz *et al.*, 1986) have used a more stringent criteria of colony formation of ≥ 2 mm diameter following 10 - 14 days in culture as indicative of colonies derived from the primitive progenitor, the HPP-CFC. Therefore, we examined HPP-CFC colony formation following ten and twelve days of incubation under the standard culture conditions for the development of colonies derived from GM-CFC and M-CFC progenitors. Since recent work by Mochizuki *et al.* (1987) demonstrated that high doses of IL 1 could serve as a "synergistic activity" (SA) with GM-CSF for the development of colonies from HPP-CFC, we also included rIL 1 in combination with rGM-CSF as a positive control for HPP-CFC stimulation in our assay.

As described in Figure 3, the number of progenitors which responded to CSF-1 alone to form colonies of < 2 mm diameter was approximately 6-fold greater than the number of progenitors which responded to rGM-CSF. Simultaneous addition of rGM-CSF and nCSF-1 to bone marrow cultures on Day 0 resulted in an increased number of large colonies (≥ 2 mm) at Day 10 (D10) with 21 colonies/ 10^5 BMP of ≥ 2 mm diameter (Figure 24A). In addition, Table 9 shows the effect of combining various concentrations of GM-CSF and CSF-1 for the generation of HPP-CFC-derived (≥ 2 mm) colonies. These data verify that the concentrations used were not in a sub-optimal dose range. The positive control, i.e., rGM-CSF and rIL 1 added simultaneously, also resulted in the generation of 11 colonies/ 10^5 BMP of ≥ 2 mm diameter (Figure 24A). This

Figure 24. Ability of rGM-CSF, CSF-1, or rIL 1 (alone or in combination) to stimulate HPP-CFC colony formation. Five $\times 10^4$ bone marrow cells were cultured in the presence of 1000 units/culture of rGM-CSF, CSF-1, or rIL 1 alone and in combination and were assayed following (A) 10 and (B) 12 days of incubation as described in the Materials and Methods. Colony formation was scored as < 2 mm or ≥ 2 mm diameter using an inverted microscope fitted with an eyepiece reticle (40X magnification). The data represent the arithmetic mean \pm SEM for 3 separate experiments.

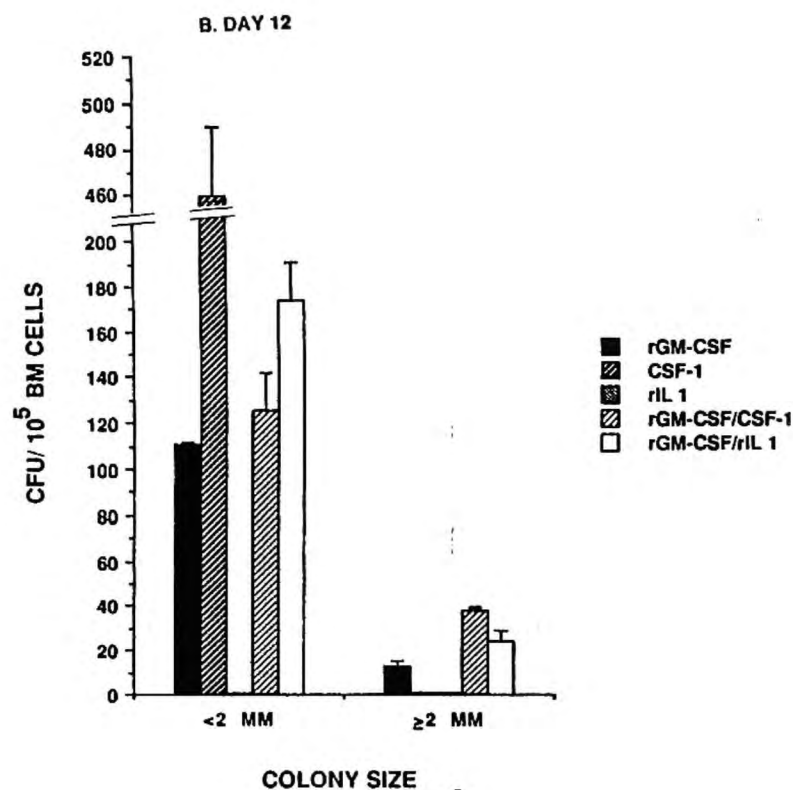
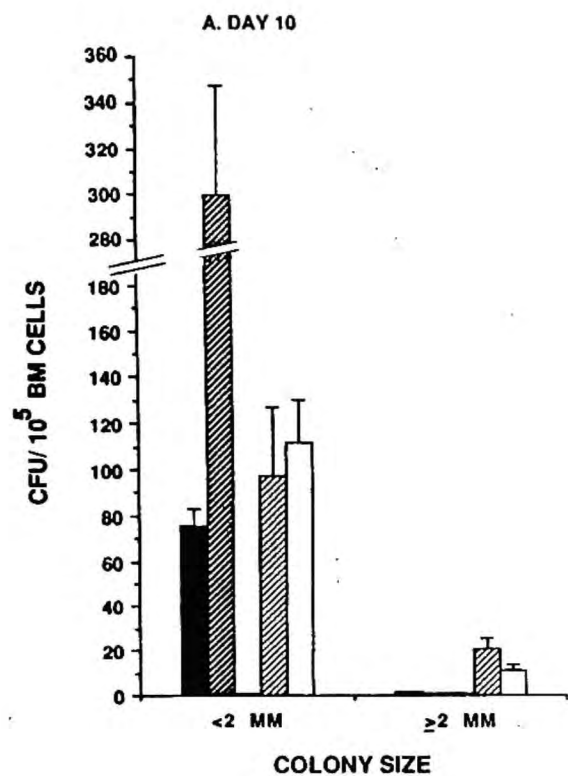


Table 9

Effect of Combined GM-CSF and CSF-1 Treatment on the Formation of Large
(> 2mm) HPP-CFC-like Colonies ^a

	<u>CSF-1 (U/CULTURE)</u>			
	<u>10</u>	<u>100</u>	<u>1000</u>	<u>5000</u>
<u>GM-CSF (U/CULTURE)</u>				
<u>10</u>	0 ± 0	0 ± 0	43 ± 26	41 ± 2
<u>100</u>	9 ± 1	33 ± 4	80 ± 3	75 ± 7
<u>1000</u>	18 ± 0	26 ± 3	56 ± 8	56 ± 6
<u>5000</u>	6 ± 3	27 ± 1	42 ± 0	46 ± 22

^a The number of HPP-CFC (> 2 mm colonies) were examined following 12 days of culture in the double agar overlay assay as described in the Materials and Methods for HPP-CFC colony formation. These data represent the mean ± one standard deviation of duplicate cultures from a single representative experiment.

difference in the ability of CSF-1 and IL 1 to synergize with 1000 U/culture of GM-CSF to form large colonies was not due to limiting concentrations of rIL 1 in the cultures since 1000 U/culture of rIL 1 gave the same number of colonies which were ≥ 2 mm in diameter as did 1 U/culture (i.e., 26 ± 7 colonies ≥ 2 mm diameter/ 10^5 BMP in cultures which received 1000 U/culture versus 21 ± 10 colonies ≥ 2 mm diameter/ 10^5 BMP in cultures which received 1 U/culture). Recombinant IL 1 alone never gave rise to colonies. Analysis of the same cultures at Day 12 (D12; Figure 24B) showed increased numbers of large colonies following simultaneous treatment with rGM-CSF and either nCSF-1 or rIL 1: rGM-CSF and nCSF-1 treatment resulted in 38 colonies/ 10^5 BMP of ≥ 2 mm diameter, while rGM-CSF and rIL 1 treatment resulted in 24 colonies/ 10^5 BMP of ≥ 2 mm diameter. It should be noted that using the ≥ 2 mm diameter criterion as indicative of an HPP-CFC-derived colony, rGM-CSF alone at D12 exhibited a limited ability to stimulate these cells (12 colonies/ 10^5 BMP). At no time over the 12 day assay period did CSF-1 alone (Figure 24A and B) or nCSF-1 and rIL 1 synergize to result in colonies of ≥ 2 mm in this assay system. Examination of either synergistic combination (i.e., rGM-CSF plus CSF-1 or rGM-CSF plus rIL 1) on Day 10 or Day 12 for colonies equal to 2 mm in diameter failed to suggest any differences in the kinetics of colony development. Each D12 colony of ≥ 2 mm diameter contained approximately 2×10^4 cells/colony, consistent with previous descriptions of HPP-CFC colonies (Bradley and Hodgson, 1979; Baines *et al.*, 1981; Bertoncello *et al.*, 1987).

Differences in colony size observed under the influence of rGM-CSF and/or either nCSF-1 or rIL 1 are illustrated in Figure 25. Photographs of colony development at D10 are shown for rGM-CSF alone (A), CSF-1 alone (B), rGM-CSF plus nCSF-1 (C), and rGM-CSF plus rIL 1 (D). The 2 mm X 2 mm grid, which is etched onto the bottom of the culture dish, serves as an internal measure of absolute colony size.

Signal separation of GM-CSF and CSF-1 in the generation of HPP-CFC-like colonies. To determine the sequence and proximity requirements for rGM-CSF and nCSF-1 to exhibit synergistic activity in the development of HPP-CFC colonies, cultures were initiated on Day 0 (D0) in either 1000 U of rGM-CSF or nCSF-1, overlaid on various times in culture with 1000 U

Figure 25. Colony formation in the presence of rGM-CSF, CSF-1, and rIL 1, alone and in combination. Photographs of representative colonies, taken from one of three separate experiments, cultured in the presence of 1000 units/culture of rGM-CSF alone (A) and HUrCSF-1 alone (B). The 2 mm X 2 mm grid, etched into the bottom of the culture dish, serves as an internal indicator of colony size.

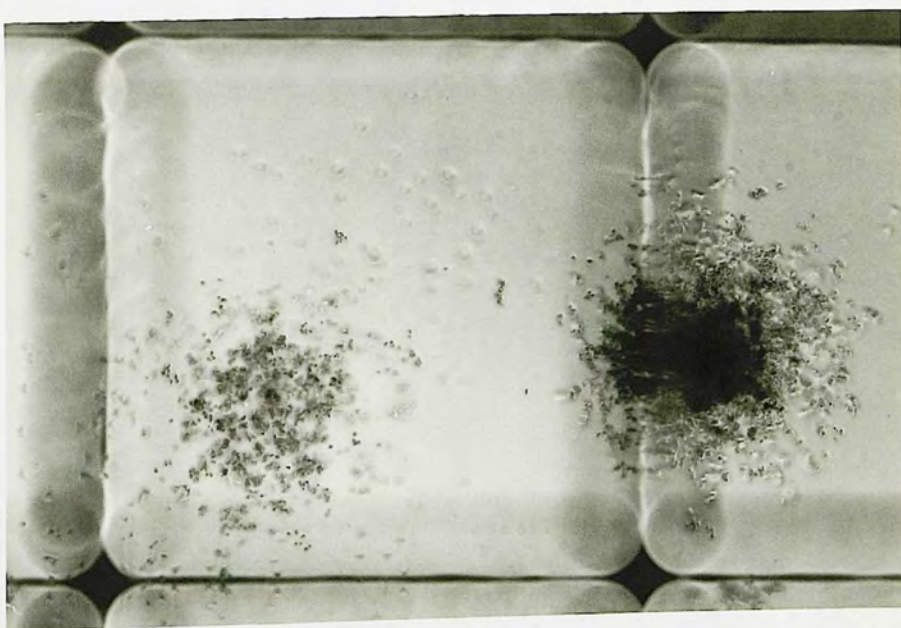
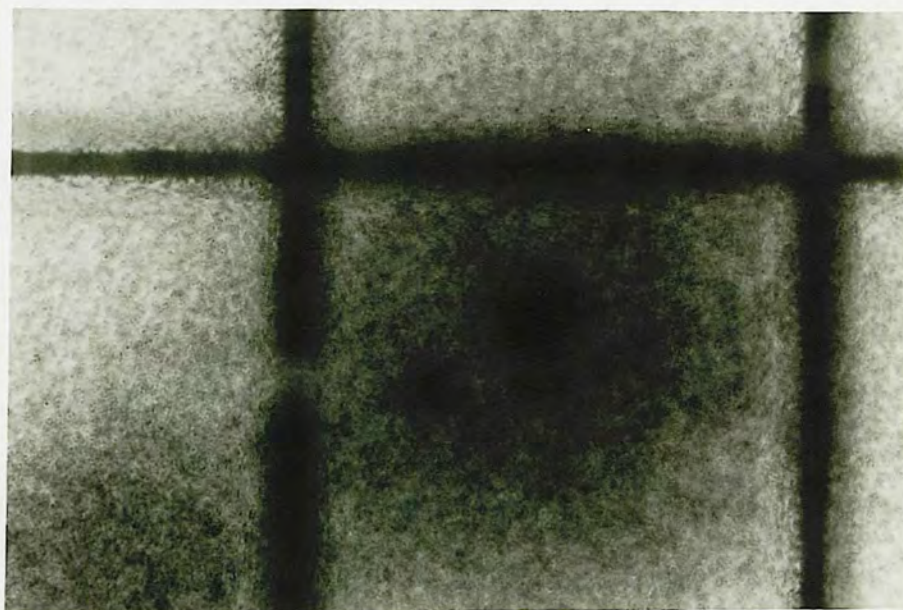
A. rGM-CSF ALONE**B. CSF-1 ALONE**

Figure 25. Colony formation in the presence of rGM-CSF, CSF-1, and rIL 1, alone and in combination. Photographs of representative colonies, taken from one of three separate experiments, cultured in the presence of 1000 units/culture of rGM-CSF and HUrCSF-1 (C) rGM-CSF and rIL 1 (D). The 2 mm X 2 mm grid, etched into the bottom of the culture dish, serves as an internal indicator of colony size.

C. rGM-CSF PLUS CSF-1**D. rGM-CSF PLUS IL 1**

of the alternate CSF, and examined 12 days after initiation of culture. Figure 26A demonstrates that in cultures initiated in the presence of rGM-CSF, nCSF-1 could be added as late as D5 and still result in reduced, but significant, HPP-CFC colony development (48 colonies/ 10^5 BMP when added simultaneously on D0 vs. 25 colonies/ 10^5 BMP when rGM-CSF was added on D0 and nCSF-1 on D5). Once again, control cultures (rGM-CSF alone on D0) resulted in only minimal HPP-CFC colony generation.

When nCSF-1-initiated cultures were examined (Figure 26B), the same trend was observed. The delayed addition of the second signal (rGM-CSF) for up to 5 days still resulted in significant HPP-CFC colony formation. However, in contrast to the findings in rGM-CSF-initiated cultures, nCSF-1-initiated cultures exhibited a greater reduction in the number of HPP-CFC colonies when the two signals were separated by 5 days (i.e., 48 colonies/ 10^5 BMP when added simultaneously on D0 vs. 12 colonies/ 10^5 BMP when the rGM-CSF was added on D5). No additional HPP-CFC colonies were detected when cultures were examined 12 days after the addition of the second CSF (Figure 27).

The ability of both sets of cultures to give rise to colonies of lower proliferative potential (< 2 mm diameter) also differed. In rGM-CSF-initiated cultures (Figure 26A), the number of colonies < 2 mm diameter remained relatively constant (approximately 85 - 100 colonies/ 10^5 BMP) in cultures which received CSF-1 as late as 3 days after the initiation of culture. In contrast, CSF-1-initiated cultures exhibited increased numbers of colonies (from 85 to 553 colonies/ 10^5 BMP) the later the rGM-CSF was added. Therefore, GM-CSF restricts the proliferation of CSF-1-responsive, low proliferative potential cells, when the two cytokines are added in close temporal proximity.

Possible contribution of GM-CSF as SA in human spleen cell conditioned media. Figure 27 demonstrates that, in the absence of NHS, natural murine or recombinant human CSF-1 could synergize with murine rGM-CSF to induce HPP-CFC-derived colonies (40 colonies/ 10^5 versus 38 colonies/ 10^5 BMP, respectively). Several additional cytokine combinations were examined to determine if human GM-CSF could be the active synergistic factor in human

Figure 26. Signal separation of rGM-CSF and nCSF-1 in the stimulation of HPP-CFC. Five $\times 10^4$ bone marrow cells were cultured in the presence of either rGM-CSF on D0 (A) or nCSF-1 on D0 (B). Cultures were then overlayed with the opposite CSF on D0 or on subsequent days. Colony formation was scored 12 days from the initiation of culture and colony diameter scored as < 2 mm or ≥ 2 mm as described in the Materials and Methods. Control cultures are cultures which received rGM-CSF only or nCSF-1 only on D0. The data represent the arithmetic mean \pm SEM for 3 - 8 individual experiments.

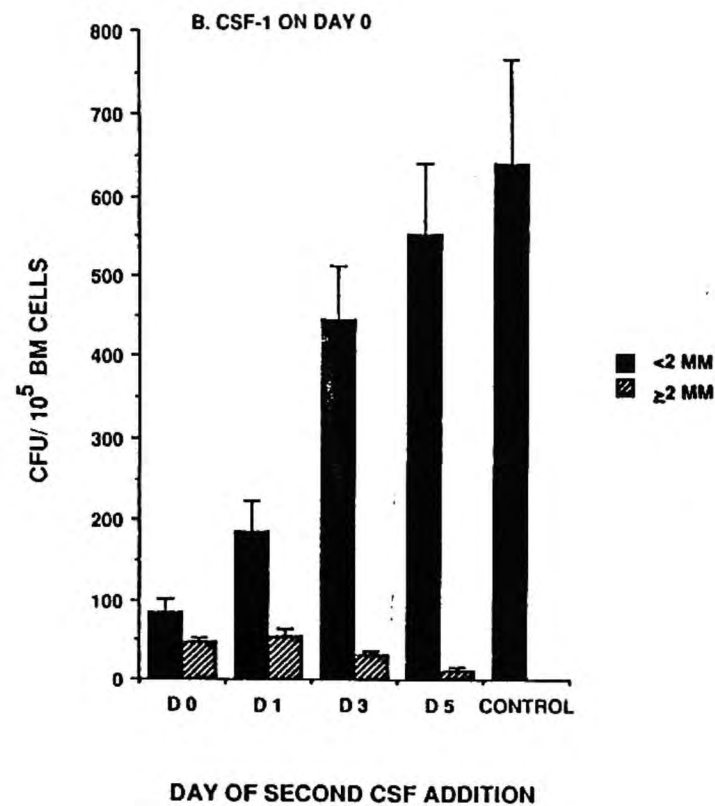
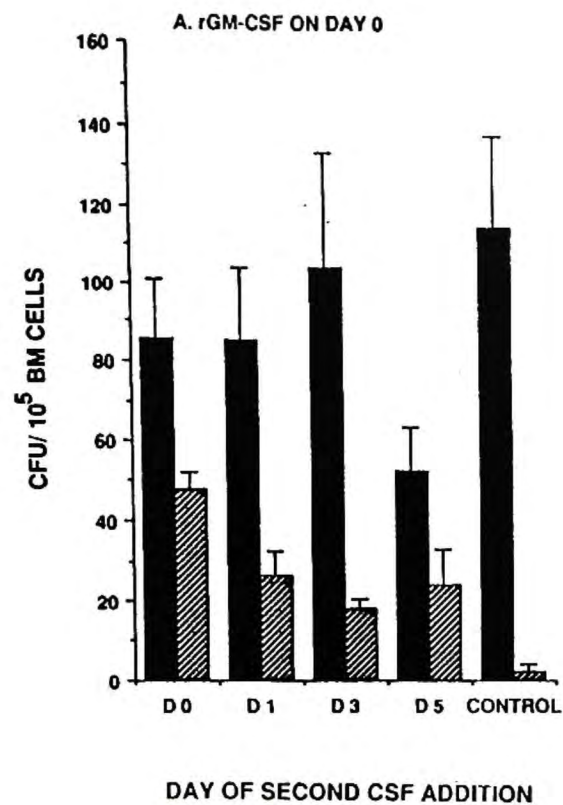
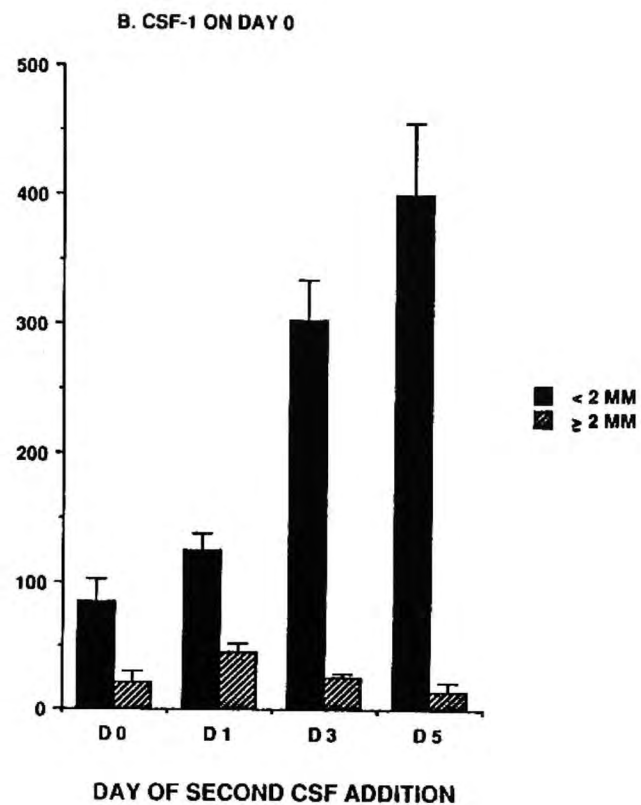
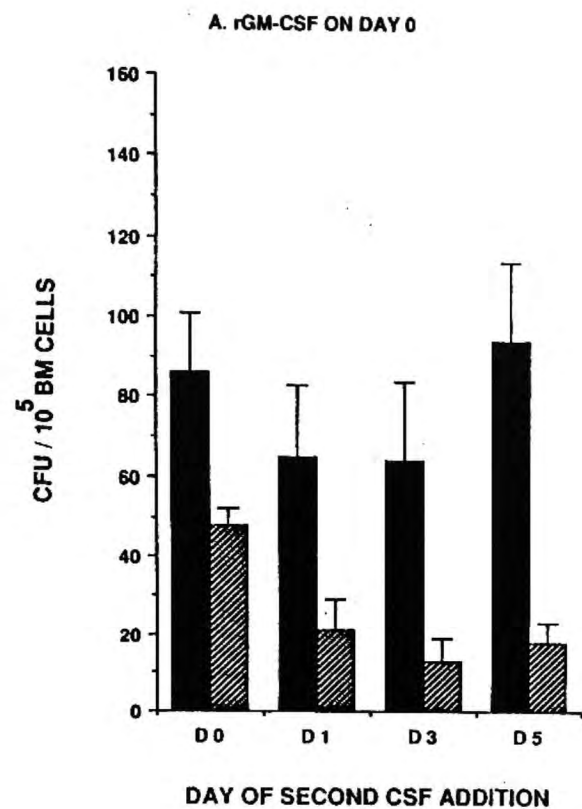


Figure 27. Signal separation of rGM-CSF and nCSF-1 in the stimulation of HPP-CFC. Five x 10⁴ bone marrow cells were cultured in the presence of either rGM-CSF on D0 (A) or nCSF-1 on D0 (B). Cultures were then overlayed with the opposite CSF on D0 or on subsequent days. Colony formation was scored 12 days from the addition of the second CSF to cultures and colony diameter scored as < 2 mm or ≥ 2 mm as described in the Materials and Methods. Control cultures are cultures which received rGM-CSF only or nCSF-1 only on D0. The data represent the arithmetic mean ± SEM for 3 - 8 individual experiments.



spleen cell conditioned medium (HSCM), the source of "synergistic activity" or SA originally described by Bradley and Hodgson (1979). Table 10 shows that human rCSF-1 failed to synergize with HSCM unless NHS were present. If human rGM-CSF were used in lieu of HSCM, even in the presence of NHS, synergy was not observed.

Examination of HPP-CFC colony formation in mice following 5-FU treatment. The original reports in the literature which describe the proliferative expansion of HPP-CFC to form colonies used bone marrow cells from 5-fluorouracil (5-FU)-treated mice as a source of progenitors responsive to CSF-1 and SA (Bradley and Hodgson, 1979). Five-FU is cytotoxic for cells which are actively cycling (Eaves and Bruce, 1979). Therefore, we next examined the HPP-CFC colony formation in bone marrow cells derived in the presence of GM-CSF and CSF-1 harvested from mice 3 days and 7 days post-5-FU treatment. These two times were chosen based on work by Bradley and Hodgson (1979) which showed maximum decreases in the number of viable cells recovered from bone marrow on these days. The yield of bone marrow cells from mice which received 5-FU 3 and 7 days earlier, represented 14% and 9%, respectively, of control yields. The cell sizing profiles of bone marrow cells harvested from normal mice, as well as from mice 3 days and 7 days post-5-FU, are shown in Figure 28. The cell size distribution of normal bone marrow (Figure 28A) shows a bimodal distribution. In contrast, bone marrow cells harvested 3 days after 5-FU (Figure 28B) exhibited a reduction in the first (smaller) peak of cells and resulted in a "shift" in the second peak to a slightly larger population. By 7 days post-5-FU, there was a single peak of small cells only (Figure 28C). Figure 29 shows that a small but significant number of progenitors remained following 5-FU treatment which were responsive to GM-CSF and CSF-1.

Examination of mature macrophages cultured in the presence of both GM-CSF and CSF-1. A preliminary characterization of the morphological and functional capabilities of mature macrophages derived from bone marrow progenitors under the influence of GM-CSF and CSF-1 in liquid culture was next examined. Table 11 shows that following 7 days in liquid

Table 10

Comparison of Synergy Between Human rCSF-1 and Human Spleen Cell Conditioned
Medium Versus Human rCSF-1 and Human rGM-CSF^a

<u>TREATMENT</u>	<u>NHS</u>	<u>CFU > 2 MM/10⁵ BMP</u>
HUrCSF-1 + HSCM	-	0 ± 0
HUrCSF-1	+	9 ± 1
HUrCSF-1 + HSCM	+	54 ± 6
HUrCSF-1 + HUrGM-CSF	+	8 ± 4

^a The ability of human rCSF-1 (HUrCSF-1) to synergize with reported sources of SA was examined in the standard assay conditions detailed in the Materials and Methods, as well as under assay conditions in which 25% normal human serum (NHS) was added to the standard culture medium. Colony formation was analyzed following 12 days.

^b Human rCSF-1 and human rGM-CSF (HUrGM-CSF) were used at a final concentration of 1000 U/culture. Human spleen cell conditioned medium (HSCM) was used at a final concentration of 10%.

^c The data represent the arithmetic means ± standard deviation of duplicate cultures from a representative experiment.

Figure 28. Cell sizing profiles of normal bone marrow cells and bone marrow cells derived from mice 3 and 7 days post-treatment with 5-FU. Bone marrow cells from mice which received an i.v. injection of 150 mg/kg of 5-FU were examined 3 days and 7 days post-injection by Coulter Channelyzer analysis using the following parameters: attenuation = 2, window width = 40, and matching = 10. Panel A represents the cell sizing profile derived from untreated mice, panel B represents the cell sizing profile derived from mice which received 5-FU 3 days prior to cell harvest, and panel C represents the cell sizing profile from mice which received 5-FU 7 days prior to harvest.

Figure 29. Quantitation of bone marrow-derived HPP-CFC colonies following stimulation by rGM-CSF and nCSF-1 in bone marrow cells derived from normal mice and mice 3 and 7 days post-treatment with 5FU. Bone marrow cells were collected as described in the Materials and Methods from one tibia and one femur per mouse. The cells were LSM-purified and cultured at 5×10^4 bone marrow cells per culture in the double agar overlay assay described in the Materials and Methods. The data represent number of colonies ≥ 2 mm which formed after 12 days of incubation and are expressed as the number of colonies per mouse (rather than the number per 10^5 viable bone marrow cells) as determined from the total viable yield of bone marrow cells obtained divided by the number of mice per group. This was done to adjust for the massive losses in viable bone marrow cells following treatment with 5-FU. The results represent the arithmetic mean \pm SEM for two separate experiments.

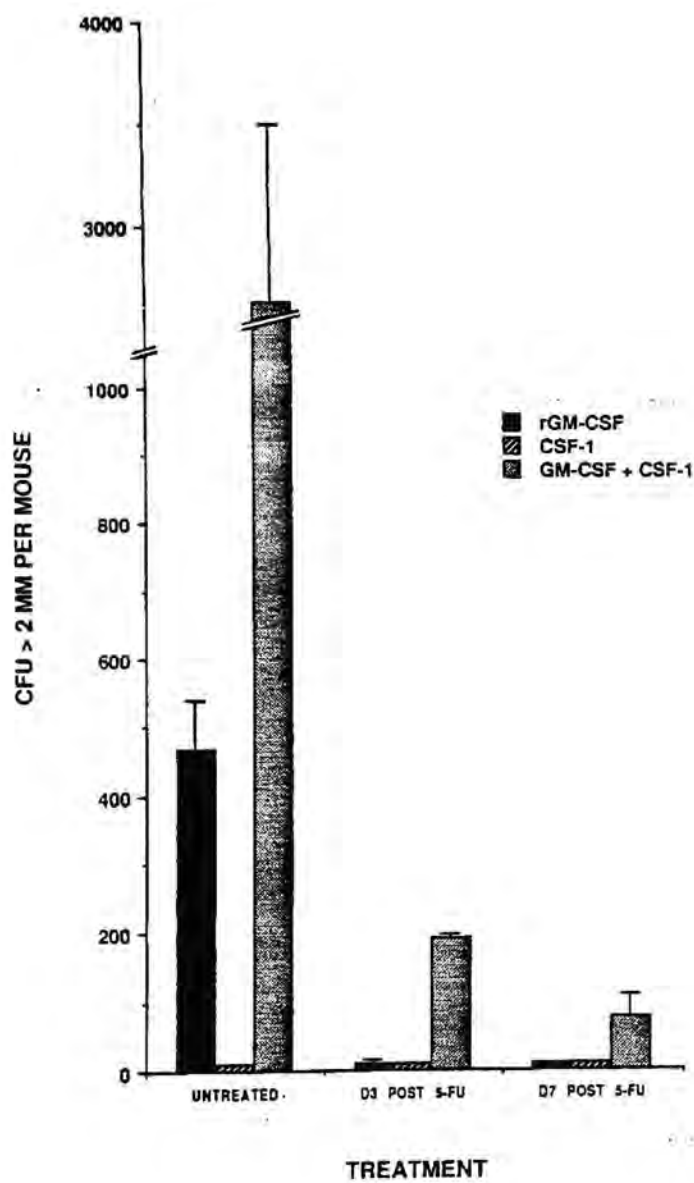


Table 11

Cell Sizing Distribution of Mature Macrophages Derived under the Influence of
both GM-CSF and CSF-1^a

CELL SIZING PROFILES (% CELLS)

<u>CELL TYPE</u>	<u>CHANNEL NUMBER</u>		
	<u>0-15</u>	<u>16-45</u>	<u>46-99</u>
rGM-CSF	41	42	17
nCSF-1	13	37	56
rGM-CSF and nCSF-1	31	46	24

^a One $\times 10^7$ C3H/HeJ bone marrow cells were cultured in the presence of 250 U/ml each of nCSF-1 and/or rGM-CSF in liquid culture as described in the Materials and Methods. Following the 7 day culture period, mature macrophages were harvested and examined for cell size distribution by Coulter Channelyzer analysis exactly as described in the Materials and Methods. The data were derived from a single representative experiment of 4 separate experiments.

^b The channel numbers correspond to the following cell size (μ diameter) ranges: channel numbers 0 - 15 = 7.0 - 9.3 μ ; channel numbers 16 - 45 = 9.4 - 11.9 μ ; and channel numbers 46 - 99 = 12.1 - 15.0 μ .

culture, mature macrophages which were cultured in the presence of both GM-CSF and CSF-1, exhibited cell sizing profiles reminiscent of GM-CSF-derived macrophages (Figure 10).

Examination of these macrophages for the early macrophage functional characteristic of Fc-mediated phagocytosis revealed that only 23% of the macrophages derived in both GM-CSF and CSF-1 exhibited Fc receptor-mediated phagocytosis and exhibited a weighted mean of 3.3 SRBC ingested per positive cell, in contrast to those derived in nCSF-1 only (93% phagocytic; 5.3 SRBC ingested per positive cell). In addition, the macrophages derived in the presence of both GM-CSF and CSF-1 exhibited intermediate sensitivity to VSV induced CPE ($32 \pm 5\%$). In the same experiments, rGM-CSF-derived macrophages exhibited $50 \pm 5\%$ CPE and nCSF-1-derived macrophages only $2 \pm 2\%$ CPE in 4 separate experiments. Further examination of these macrophages with regard to their Ia antigen expression (Table 12) revealed high levels of total Ia antigen expression which did not increase in the presence of rIFN- γ .

Thus, the macrophages which were derived in liquid culture in the presence of both GM-CSF and CSF-1 exhibited functional characteristics which more closely resembled those of macrophages cultured in the presence of GM-CSF alone, i.e., heterogeneity with regard to cells with small diameters as shown by the cell size distribution, low Fc receptor-mediated phagocytic capacity, relative sensitivity to VSV-induced CPE, and high basal Ia antigen expression.

Table 12

Ia Antigen Expression in Mature Macrophages Derived under the Influence of
both GM-CSF and CSF-1^a

TOTAL Ia ANTIGEN EXPRESSION (OD₄₉₀)^b

<u>CELL TYPE</u>	<u>DAY 1</u>		<u>DAY 2</u>	
	<u>MEDIUM</u>	<u>+IFN-γ</u>	<u>MEDIUM</u>	<u>+ IFN-γ</u>
rGM-CSF	0.587 \pm 0.084	0.625 \pm 0	0.577 \pm 0.034	0.633 \pm 0.032
nCSF-1	0.432 \pm 0.046	0.542 \pm 0.020	0.415 \pm 0.042	0.627 \pm 0.021
rGM-CSF and nCSF-1	0.592 \pm 0.046	0.596 \pm 0.021	0.600 \pm 0.021	0.613 \pm 0.028

^a One $\times 10^7$ C3H/HeJ bone marrow cells were cultured in the presence of 250 U/ml each of nCSF-1 and rGM-CSF in liquid culture as described in the Materials and Methods. Following the 7 day culture period, mature macrophages were harvested and re-plated at 2×10^5 cells per well for each assay.

^b Total Ia antigen expression was measured by ELISA exactly as described in the Materials and Methods and the data represent the arithmetic mean \pm SEM for 3 - 5 separate experiments.

DISCUSSION

Heterogeneity within mature macrophage populations has been hypothesized to reflect cell populations at various differentiation states along the differentiation pathway which leads to the development the fully mature or "activated" macrophage (Cohn, 1978). Most of the early work which led to a definition of functional or topographical association with different states of activation involved examination of heterogeneous populations of macrophages present in the peritoneal cavity of untreated mice versus mice which had received various stimulants or eliciting agents intraperitoneally. Work by a number of investigators (Lazdin *et al.*, 1975; Beelen *et al.*, 1978; Bursucker *et al.*, 1982) demonstrated that resident peritoneal macrophages exhibited different peroxidase and 5'-nucleotidase activities than peritoneal exudate macrophages. In these studies, resident macrophages were shown to exhibit a high degree of 5'-nucleotidase activity and peroxidase activity which was localized to the nuclear envelope, in contrast to low 5'-nucleotidase activity and peroxidase activity which was localized to cytoplasmic granules in exudate macrophages. These findings provided the first evidence that biochemical differences could be utilized as "markers" for the differentiation state of a macrophage. In addition, the observations that peritoneal macrophages elicited by various agents differ with respect to their expression of Fc receptors, C3b receptors, and Ia antigens (Bianco *et al.*, 1975; Spitalny, 1981; Beller and Ho, 1982), and that macrophages isolated from various organs also exhibit differential Ia expression (Cowing *et al.*, 1978), added to the experimental evidence in support of macrophage heterogeneity as a function of differentiation. Finally, macrophages could be activated *in vivo* by administration of potent "macrophage activating agents" (e.g., Bacillus Calmette Guerin) or treated *in vitro* with lymphokines plus endotoxin, to induce macrophages which were "fully activated" as assessed by their microbicidal or tumoricidal activity (Nogueria *et al.*, 1978; Hibbs, 1976).

This heterogeneity, as assessed by biochemical, functional, and differentiation markers, has prompted much speculation and debate as to the nature, location, and generation

of these subpopulations of macrophages. Several theories have been proposed to address this heterogeneity and include (i) major alterations at the site of an inflammatory insult (Van Furth, 1980), and (ii) stimulation of functionally unique subsets of progenitors from the bone marrow which may home to specific organs (Bursucker and Goldman, 1983). In the former case, Van Furth (1980) proposed that the bone marrow acted solely as a depot for the release of macrophages into the inflamed site, where they were acted upon by lymphokines released in response to the inflammatory insult. The latter hypothesis has been explored principally through the use of crude preparations of Colony Stimulating Factors (CSFs) to allow for the clonal expansion of progenitor cells within the bone marrow, as well as those within peripheral organs. This approach has been instrumental in the examination of the question of heterogeneity on a progenitor level, in contrast to an analysis of mature macrophage phenotypes within a complex mixture of cells.

The goal of this dissertation was to focus on the question of development of macrophage heterogeneity through the use of bone marrow-derived progenitors stimulated with highly purified or recombinant preparations of CSF-1 or GM-CSF. Both forms of CSF have been shown to act upon bone marrow progenitors to give rise to mature macrophages. However, these two species of CSF share no sequence homology and bind to distinct receptors on cells (reviewed by Metcalf, 1986). Thus, the existence of two forms of the same lineage-specific differentiation factor led to questions which address the *raison d'être* for this apparent cytokine redundancy. Do two discrete forms of CSF exist as an evolutionary "failsafe" system for the maintenance of macrophage populations? One could argue that the macrophage represents a major line of defense in primary host resistance to infection, and therefore, the two distinct differentiation factors evolved to ensure preservation of such an important cell lineage. Fibroblasts represent the principal source of CSF-1 (reviewed by Metcalf, 1985), and as such, one might expect this factor to be present throughout the body. In contrast, the primary source of GM-CSF is felt to be stimulated T cells (reviewed by Metcalf, 1985). Thus, the availability of GM-CSF might be more restricted to areas, such as the spleen,

bone marrow, thymus, and lymph nodes. The presence of these two factors allows for the maintenance of basal levels, as well as, provides for the rapid generation of high concentrations of CSFs during an inflammatory response in the periphery and in the microenvironment. Alternatively, the existence of two forms of CSF, both capable of giving rise to macrophages, might suggest a mechanism by which functional heterogeneity could be induced. Through a side by side comparison, we have examined the number of bone marrow progenitors which respond to CSF-1 and GM-CSF in vitro, as well as the functional and differentiative characteristics of the mature macrophages which develop from appropriately stimulated progenitors.

Progenitor Studies

The first question which was addressed was, "Are the macrophage progenitors which respond to GM-CSF and CSF-1 equal in number?" Experiments carried out to address this question revealed that by Day 10 in culture, the number of bone marrow progenitors which respond to CSF-1 to form colonies was approximately six-fold greater than the number of colonies which developed in response to GM-CSF. This difference was observed over a wide dose range and was detected in both LPS-responsive and LPS-hyporesponsive mouse strains (Figure 3).

The latter findings are in contrast to those of MacVittie and Weinberg (1980) which demonstrated that there were approximately 2-fold greater numbers of M-CFC in the LPS-hyporesponsive (*Lps*^d) C3H/HeJ mouse strain than in the LPS-responsive (*Lps*ⁿ) C3HeB/FeJ mouse strain. The apparent differences between the findings of these investigators and our findings (using the LPS-responsive C3H/OuJ mouse strain) may be attributed to a number of different parameters, the first of which is usage of different LPS-responder strains of mice. The C3H/OuJ mouse is more closely related genetically to the C3H/HeJ mouse strain (Dr. B. Taylor, personal communication) and, therefore, may serve as a better control strain in

these studies. In addition, previous findings suggest that the C3HeB/FeJ strain possesses a defect in the ability to control replication of *S. typhimurium* in their spleen (O'Brien *et al.*, 1983), a previously reported macrophage function (O'Brien *et al.*, 1979). Although this defect is unrelated to LPS-responsiveness (Eisenstein *et al.*, 1982), the use of this mouse strain as a control may be questionable and might have contributed to the differences in the findings.

Alternatively, the differences in numbers of CSF-1-responsive progenitors in *Lps*^{II} versus *Lps*^d strains reported by MacVittie and Weinberg (1980) versus our findings may have been related to the use of crude culture supernatants from L929 fibroblasts as a source of CSF-1 in the former study. These and many other early studies utilized such supernatants as a source of CSF-1, not recognizing the potential role of contaminating IFN- α/β in the crude CSF-1 preparations. Since these studies were carried out, it has been well-documented that IFN- α/β can act as an antagonist of CSF-1 (Klimpel *et al.*, 1982; Moore *et al.*, 1984; Raefsky *et al.*, 1985). In our studies, we have determined that there are equal numbers of CSF-1-responsive progenitors present in LPS-responsive and hypo-responsive strains using highly purified or recombinant preparations of CSF-1 shown to be devoid of this contaminating cytokine.

Lastly, one additional explanation for the contradictory findings may be that the number of responsive progenitors observed in LPS-responsive strains can vary depending on the degree of *in vivo* stimulation prior to *in vitro* culturing. Many environmental pathogens, as well as LPS, have been shown to increase IFN- α/β levels, and the IFN levels of germ-free, pathogen-free, and conventional mice have been shown to differ dramatically (Galabrue *et al.*, 1985). Thus, increased levels of interferon induced *in vivo* may reduce the number of progenitors which are capable of responding to CSF-1. This hypothesis was directly addressed by Goris *et al.*, (1985) in which experimentally contaminated pathogen-free and germ-free mice exhibited lowered numbers of GM-CFC in their bone marrow. While there is no mention of special care taken to ensure cleanliness of the responder mice in the report by MacVittie and Weinberg, we have taken extreme care to purchase mice from a virus-free source, keep them in the animal facility for a minimum amount of time, maintain them on autoclaved food

and acid water, and house them in laminar flow hoods with filter cage tops. Therefore, our inability to demonstrate reduced numbers of M-CFC or GM-CFC in our responder mice may reflect the cleanliness of the mice and as a consequence, a reduced state of *in vivo* immune stimulation, thus allowing for uninhibited colony formation.

When the kinetics of colony formation in response to CSF-1 or GM-CSF were analyzed, the numbers of progenitors which responded to either CSF preparation at Day 5 were found to be comparable, but continued to increase, with time, only in those cultures which contained CSF-1 (Figure 4). The increase in the number of CSF-1-derived colonies over the culture period may indicate the existence of a separate population of CSF-1-responsive progenitor cells which can be stimulated at a later time in culture. This possibility was originally put forth by the work of Lin and Stewart (1974) and MacVittie and McCarthy (1977) who demonstrated that, in the peritoneal cavity and in the lymph nodes, there exist cells which can respond to CSF-1 contained in pregnant mouse uterine extract (PMUE) to form colonies after a significant "lag time" (10 - 14 days) in culture. These progenitors were found to be restricted to the generation of cells of the macrophage lineage, and have a slower rate of colony formation after addition of CSF to cultures (MacVittie and McCarthy, 1977). However, examination of the number of CSF-responsive cells following delayed addition of CSF, as described by MacVittie (1979), indicated that this colony forming ability was significantly reduced in both CSF-1 and GM-CSF cultures following a 24 hour period of CSF deprivation (50% reduction in GM-CSF cultures and 60% in CSF-1 cultures; Figure 6). In cultures which were CSF-deprived for 3 - 4 days, little colony formation was observed when examined 10 days from the initiation of culture. These findings are consistent with those reported for CSF-deprivation of bone marrow progenitors reported by Lin and Stewart (1974) which showed that following 24 hours of CSF-deprivation, there was a 60% reduction in colony forming capabilities. This reduction was more dramatic by D4 of deprivation, in that only 10% of the progenitors were still capable of colony formation. Although allowing CSF-1-deprived cultures to incubate for as long as 10 days after the time of addition led to the formation of approximately $100 \text{ colonies}/10^5 \text{ input cells}$, this is

far below the additional number of colonies which arise in CSF-1-treated cultures between Days 5 and Day 10 (approximately 350 colonies/ 10^5 input cells). Thus, a population of cells which responds late in culture cannot account for the increase in colonies observed between Days 5 and 10 in CSF-1 cultures.

An alternate explanation for the appearance of additional colonies in the CSF-1-stimulated cultures may be that products produced by CSF-1-stimulated bone marrow cells early in culture induce receptivity of additional progenitors for CSF-1 (Bender *et al.*, 1986; Broxmeyer, 1986). In this regard, exposure of bone marrow progenitors to Hemopoietin-1 (Bartelmez and Stanley, 1985; Mochizuki *et al.*, 1987), Hemopoietin-2 (Bartelmez *et al.*, 1985), or very low concentrations of IFN- α/β (Moore and Rouse, 1983), have been shown to result in increased capacity of bone marrow progenitors to proliferate in response to CSF-1. The demonstration that CSF-1-derived macrophages produce more IFN- α/β during development than GM-CSF-derived macrophages (Table 5) strengthens the possible role for low levels of endogenous IFN- α/β as a "competence" factor in the generation of additional CSF-1-responsive progenitors with time in culture. Since GM-CSF-derived macrophages produce less than 1.0 U/ml of IFN- α/β endogenously (Table 5), perhaps supplementing GM-CSF cultures with various concentrations of IFN- α/β may result in the generation of additional GM-CSF-responsive cells.

In addition to these "competence" factors (Bender *et al.*, 1986), the endogenous production of hematopoietic growth factors by maturing macrophages must also be considered as a possible mechanism for the generation of increased colony formation in the CSF-1-derived cultures (Ralph *et al.*, 1986; Rich, 1986; Horiguchi *et al.*, 1987). Although the ability to detect endogenous CSF production in macrophage supernatants is technically difficult, due to the autocrine re-utilization of this cytokine, a number of investigators have demonstrated CSF activity in supernatants from stimulated macrophages (Sullivan *et al.*, 1985; Herrmann *et al.*, 1986; Rambaldi *et al.*, 1987; Warren and Ralph, 1986), as well as, CSF-specific mRNA in macrophages following stimulation with either CSF-1 or GM-CSF (Horiguchi *et al.*, 1986; Horiguchi *et al.*, 1987; Thorens *et al.*, 1987). Thus, the potential role of endogenous CSF

production in the generation of the increased colony formation observed between D5 and D10 in culture will require further investigation. The elucidation of this as a mechanism awaits the availability of high-titered neutralizing anti-murine CSF antibodies. Although anti-murine CSF-1 and anti-murine GM-CSF antibodies have been made available to us, the quantities of these reagents has been extremely limited. Neutralization of CSF-induced colony formation requires pre-treatment of CSF-containing preparations prior to the addition of responsive cells (Mochizuki *et al.*, 1986), as well as inclusion of the antisera in individual cultures. In addition, this mode of neutralization is not highly predictable when one is attempting to neutralize the action of a cytokine produced in close proximity to cells in the soft agar assay.

The reduced numbers of colonies measured in GM-CSF- versus CSF-1-derived cultures may also reflect differences in the stability of GM-CSF versus CSF-1 over the 10 day culture period. If GM-CSF were less stable, the degradation of this material could account for a lowered colony-forming response. Although we do not have information as to the stability of these factors in our assay system, supplementation of GM-CSF progenitor cultures with an additional 2000 U/culture of GM-CSF on Day 5 did not increase the number of colonies formed on Day 10. In addition, the slight decrease in colony formation observed in GM-CSF cultures between Day 5 and Day 7 may reflect the degradation of early neutrophilic colonies which have been shown by a number of investigators to develop in the presence of high concentrations of GM-CSF (Lee and Wong, 1980; Metcalf, 1980), rather than the destruction of GM-CSF.

In addition to the possible lack of "recruitment factors" produced by developing GM-CSF-1-derived macrophages, these macrophages were shown to produce high concentrations of PGE (Table 7). Prostaglandins have been implicated by a number of investigators to be inhibitory for CSF-induced colony formation (Kurland *et al.*, 1978a; Kurland *et al.*, 1978b; Gentile and Pelus, 1987). Gentile and Pelus (1987) demonstrated that *in vivo* injection of mice with PGE (0.001 μ g/mouse) inhibited colony formation in response to CSF-1 contained in L929 conditioned medium. These authors hypothesized that PGE treatment may

prevent a new population of progenitor cells from entering into S-phase, which leads to a depletion of cycling progenitors. The role of PGE production in GM-CSF cultures as a possible inhibitor of colony growth will require further investigation. Additional studies which utilize inhibitors of PGE production, such as indomethacin, may provide insights into the possible inhibitory role of PGE production in GM-CSF cultures during colony development, and may explain the six-fold difference in colony formation observed in CSF-1 versus GM-CSF cultures.

Taken collectively, our findings indicate that the progenitor population which responds to CSF-1 is proliferatively more expansive and may not be completely identical to that which responds to GM-CSF. It is important to note that the greater number of CSF-1-responsive progenitors is not simply an artifact of the soft agar assay. Rather, the differences observed at the progenitor level were also reflected in the yields of mature macrophages derived in liquid culture (Figure 8).

Given the numerous reports in the literature as to the extensive heterogeneity within the bone marrow progenitor population (Haskill *et al.*, 1970; Williams and Jackson, 1977; Bol *et al.*, 1979; Bradley and Hodgson, 1979; Bertoncello *et al.*, 1986; McNiece *et al.*, 1986; Moore *et al.*, 1986; Bertoncello *et al.*, 1987), we sought to determine whether GM-CSF and CSF-1 were acting on the same population of progenitors or an overlapping population. It was our hope that by examining colony formation in cultures stimulated with both GM-CSF and CSF-1, we would be able to determine if these two cytokines were acting on separate populations of progenitors. However, under these conditions, the development of large (≥ 2 mm) colonies in the presence of both CSFs was an unexpected finding. Although individual species of CSF have been described as giving rise to lineage-specific populations (reviewed in Williams and Jackson, 1977; McNiece *et al.*, 1986), it is clear that these factors can act synergistically when in combination with other cell-derived factors and result in the development of large colonies with high proliferative potential and have been referred to as HPP-CFCs (Bradley and Hodgson, 1979; Baines *et al.*, 1981; Kriegler *et al.*, 1982; Bartelmez *et al.*, 1985; Bartelmez and Stanley, 1985; Bertoncello *et al.*, 1986; Koike *et al.*, 1986; McNiece *et al.*, 1986; Stanley *et al.*, 1986; Beck *et al.*,

1987; Bertoncello *et al.*, 1987; Mochizuki *et al.*, 1987; Pluznik *et al.*, 1987; Zucali *et al.*, 1987).

The methods used to demonstrate the synergistic activities of a variety of cytokines differ markedly from study to study: colony formation in soft agar (Bol *et al.*, 1979; Bradley and Hodgson, 1979; Bertoncello *et al.*, 1986; McNiece *et al.*, 1986; Bertoncello *et al.*, 1987), inclusion of a variety of sera in the culture medium (Bol *et al.*, 1979; Bradley and Hodgson, 1979; Baines *et al.*, 1981; Madonna and Vogel, 1985; Hagan *et al.*, 1985; McNiece *et al.*, 1986; Schwartz *et al.*, 1986), radiolabeled cytokine-receptor interaction (Bartelmez *et al.*, 1985; Bartelmez and Stanley, 1985; Stanley *et al.*, 1986), as well as proliferative responses measured by thymidine incorporation (Mochizuki *et al.*, 1987). In particular, a spectrum of methodologies have been utilized to study the expansion of HPP-CFC progenitors in soft agar (Bradley and Hodgson, 1979; Baines *et al.*, 1981; Madonna and Vogel, 1985; Hagan *et al.*, 1985; McNiece *et al.*, 1986; Schwartz *et al.*, 1986), most of which require an enrichment for HPP-CFC by 5-FU treatment, as well as the presence of high concentrations of normal human serum (in addition to fetal calf and horse serum). Since the original purpose of this project was to examine the populations of progenitors (GM-CFC or M-CFC) responsive to rGM-CSF or CSF-1, respectively, we used concentrations of individual CSFs shown to be in CSF excess for progenitor responsiveness under culture conditions which did not include normal human serum (Figure 3). These concentrations of GM-CSF and CSF-1 were not suboptimal for HPP-CFC stimulation, as evidenced by the data in Table 9. Figures 24 and 25 demonstrate that there were significantly greater numbers of HPP-CFC colonies in the combined presence of rGM-CSF and CSF-1 than in the combined presence of rGM-CSF and rIL 1 and that very few HPP-CFC colonies arise in the presence of rGM-CSF alone. These differences were observed both at D10 and D12.

In addition, the relative ability of rGM-CSF to act synergistically with CSF-1 in the expansion of HPP-CFC progenitors was also observed in bone marrow cells following treatment of mice with 5-FU (Figure 29). These findings are consistent with those reported by Bradley and Hodgson (1979; 1985) which showed the existence of HPP-CFC progenitors that were responsive to PMUE as a source of CSF-1, in combination with "synergistic activities" (SAs)

from a variety of sources, i.e., human spleen cell conditioned medium (HSCM), human placental conditioned medium, and conditioned medium from WEHI-3 cell lines. These subpopulations of progenitors were present in both untreated mice and mice treated with 5-FU. However, differences in their kinetics of appearance after administration of 5-FU, as well as the number of HPP-CFC colonies which formed after 5-FU (compared to control mice) illustrated several major points: (i) the existence of multiple SAs for the stimulation of HPP-CFC colony formation in combination with CSF-1, and (ii) the existence of multiple subsets of HPP-CFC which can be characterized based on the kinetics and percent of normal HPP-CFC development of bone marrow recovery following treatment of mice with 5-FU.

The demonstration by Bradley and co-workers (1979, 1985) of possibly three distinct HPP-CFC progenitors is relevant to the characterization of the population of HPP-CFC shown herein to respond to stimulation by GM-CSF and CSF-1. The repopulation kinetics of GM-CSF and CSF-1-responsive HPP-CFCs, following 5-FU treatment, are similar to that reported for those HPP-CFC responsive to PMUE and WEHI-3. In both cases, a dramatic decrease in HPP-CFC was observed at D3 when compared to untreated bone marrow (Figure 28; Bradley *et al.*, 1985). However, unlike the HPP-CFC responsive to CSF-1 and the SA in WEHI-3, the HPP-CFCs responsive to GM-CSF and CSF-1 are not significantly increased by D7. Therefore, the findings reported herein demonstrate a fourth population of HPP-CFC which can respond to GM-CSF and CSF-1 and exhibits bone marrow repopulation kinetics which have not been previously reported.

The ability of GM-CSF and CSF-1 to synergize requires additional investigation to determine whether this synergy is a direct action of GM-CSF and CSF-1 on HPP-CFC or is an indirect effect mediated through the subsequent production of additional factors. It is possible that CSF-1 may serve as the SA for GM-CSF due to its previously reported capacity to induce IL 1 in macrophage cultures (Moore *et al.*, 1980). However, this mechanism seems unlikely since the concentration of rIL 1 used in this study (1000 U) to synergize with rGM-CSF gave the same number of colonies ≥ 2 mm in diameter as seen when 1.0 U of rIL 1 was used to synergize with

rGM-CSF. Thus, even providing a gross excess of the putative intermediate (i.e., IL 1) failed to compensate fully for the differences in "HPP-CFC" colony number observed following stimulation with rGM-CSF and CSF-1. However, until a highly potent anti-murine IL 1 antibody reagent becomes available, the potential role of macrophage-derived IL 1 in the proliferative expansion of HPP-CFC in response to rGM-CSF and CSF-1 will remain an open question.

Following the observation that HPP-CFC-like colonies formed in cultures which received both murine rGM-CSF and murine or human CSF-1, we investigated the possibility that human GM-CSF was the active SA in HSCM, since the latter was previously reported to synergize with CSF-1 (Bradley and Hodgson, 1979). The data in Table 10 indicate that human GM-CSF is apparently not the active factor in HSCM, consistent with the reported inability of human GM-CSF to cross species barriers to stimulate murine bone marrow progenitors (Metcalf, 1986). In addition, Table 10 shows that in order for CSF-1 to synergize with HSCM, NHS had to be present, consistent with original studies which employed HSCM as a source of SA (Bradley and Hodgson, 1979).

To characterize further the synergy between rGM-CSF and CSF-1, we examined the temporal requirements for rGM-CSF and CSF-1 in the expansion HPP-CFC progenitors. In cultures initiated with rGM-CSF (Figure 26), the addition of an SA, in the form of CSF-1, could be delayed as long as 5 days and yet still result in the generation of HPP-CFC colonies, although the absolute number observed was approximately halved. The number of low proliferative cells which developed (< 2 mm) under these conditions resembles that derived under the influence of rGM-CSF alone. However, when CSF-1 is added prior to rGM-CSF (Figure 26), greater numbers of cells responded to form low proliferative potential (< 2 mm) colonies, with a concomitant reduction in the number of HPP-CFC colonies. This effect is most obvious when the second signal, supplied by rGM-CSF, is delayed by ≥ 3 days. Under these conditions, an increased number of low proliferative potential colonies formed (450 - 540 colonies/ 10^5 BMP). These findings indicate that in rGM-CSF-initiated cultures, the expansion of CSF-1-responsive progenitors to form colonies of < 2 mm diameter is restricted, i.e., the ability of CSF-1 to function

at its full potential appears to be limited to its role as an SA for rGM-CSF (Figure 26A). This inhibitory effect of GM-CSF on CSF-1-induced proliferation confirms previous findings of Hume *et al.* (1987) in which bone marrow cells from CBA/H mice cultured in the presence of GM-CSF and CSF-1 exhibited one-third the proliferative response, as measured by ^3H -thymidine uptake, of that induced by CSF-1 alone.

Two pieces of information support the hypothesis that CSF-1 and GM-CSF may act on an overlapping population of progenitors: (i) the previous demonstration that during colony formation, in response to either rGM-CSF or CSF-1, there is a time in culture at which progenitor numbers responsive to GM-CSF and CSF-1 are equal and (ii) the observation that the total number of colonies which form in response to simultaneous addition of rGM-CSF and CSF-1 is reminiscent of that observed in cultures treated with rGM-CSF alone. Thus, the number of responsive colonies which develop following simultaneous addition of CSF-1 and GM-CSF suggests that CSF-1 activity is somehow limited in the presence of rGM-CSF. However, when CSF-1 is given a "head start", the controlling/inhibitory effect of rGM-CSF on low proliferative potential progenitors is lost while the SA is retained. The mechanism of this inhibition remains to be determined, but may bear on many of the proposed mechanisms presented earlier in this discussion with respect to the potential role of IFN- α/β or PGE in the limited colony formation observed in GM-CSF cultures (Figure 3).

The findings presented in this section suggest that a population of early progenitors exists which responds to the synergistic signals provided by rGM-CSF and CSF-1 to proliferate as "HPP-CFCs" without enrichment by 5-FU or the presence of NHS. However, the exact relationship of the "HPP-CFC" derived under the conditions reported herein, to the HPP-CFC population originally reported by Bradley and Hodgson (1979), remains to be determined. One possibility is that the population of progenitors which responds in our system to CSF-1 and GM-CSF represents a unique HPP-CFC subpopulation, consistent with the growing body of evidence that multiple populations of HPP-CFCs exist, each with their own distinct requirements for proliferative expansion (Bertoncello *et al.*, 1986; McNiece *et al.*, 1986;

Bertoncello *et al.*, 1987). Alternatively, the progenitor population described herein may represent a cell type which has differentiated from the primitive HPP-CFC originally described by Bradley and Hodgson. It might be hypothesized that in this new differentiation state, the HPP-CFC retains high proliferative potential, but now has new signal requirements for proliferative expansion.

Studies on the functional capacities of mature macrophages derived under the influence of GM-CSF or CSF-1

The second level at which the question of cytokine redundancy was examined centered on the functional and differentiative capabilities of mature macrophages which developed under the influence of GM-CSF, CSF-1, or both. The establishment of liquid culture procedures by Warren and Vogel (1985a), and the subsequent modifications to this procedure which are described herein, allowed for further examination of the mature macrophages which develop from GM-CSF-responsive and CSF-1-responsive progenitors. Although both populations were 100% mononuclear, the two populations differed with respect to cell size and degree of vacuolization. CSF-1-derived macrophages were a larger and more homogeneous population of large macrophages when compared to the broader spectrum of cell sizes which develop under the influence of GM-CSF (Figure 10), consistent with the early findings of Lee and Wong (1980) using crude CSF-containing preparations. Thus, with respect to cell size, CSF-1-derived macrophages represent a more differentiated population. However, the high degree of vacuolization associated with GM-CSF-derived macrophages is inconsistent with the notion, based on cell size, that these macrophages represent a less-differentiated subpopulation. The heterogeneity within this population of macrophages may reflect subpopulations of macrophages with specialized functions, i.e., perhaps the smaller macrophages express high Ia, while the highly vacuolated macrophages are highly tumoricidal. These morphological

characteristics were the first indication that these two macrophage populations differed with respect to their state of differentiation.

Non-specific and Fc receptor-mediated phagocytosis. In 1980, Neuman and Sorg demonstrated that one of the first functional properties which macrophages acquire during their development is that of phagocytosis. The earliest phagocytic capacity acquired during macrophage differentiation is that of non-specific, non-receptor-mediated phagocytosis. With increasing differentiation, macrophages develop an additional, more specific mechanism of phagocytosis via Fc receptors (Neuman and Sorg, 1980). In the studies described herein, macrophages derived from bone marrow progenitors under the influence of the two CSFs were compared with regard to these two differentiation markers. Although both bone marrow-derived populations possessed these early functional markers, they differed in the magnitude of their phagocytic capabilities. The CSF-1-derived macrophages exhibited a greater capacity for both non-specific and Fc receptor-specific phagocytosis in comparison to GM-CSF-derived macrophages.

Production of endogenous IFN- α/β . There exists extensive documentation in the literature to indicate that Fc-receptor expression can be up-regulated by IFN- α/β and result in an increased capacity to phagocytose opsonized particles (reviewed by Friedman and Vogel, 1983). Given the findings presented herein (i.e., that GM-CSF-derived macrophages exhibited a reduced Fc-mediated phagocytic capacity when compared to CSF-1-derived macrophages), and the evidence in the literature that endogenous levels of IFN- α/β play a major role in Fc receptor expression (Moore *et al.*, 1984; Warren and Vogel, 1985a), we hypothesized that perhaps a major difference between these two populations of macrophages resided in the ability to produce IFN- α/β endogenously. Direct examination of the endogenous IFN- α/β production by these two populations would provide support for this theory. However, the conventional antiviral assay used to measure excreted IFN activity was found not to be adequately sensitive to test this hypothesis directly. Based on the well-documented role of IFN in antiviral activity (reviewed by Gresser, 1984), GM-CSF- and CSF-1-derived macrophages

were compared for sensitivity or resistance to viral infection. The observed viral sensitivity of GM-CSF-derived macrophages versus the refractoriness of CSF-1-derived macrophages to VSV infection further supported the possibility that differences in IFN production between these two macrophage populations might exist. Confirmation of this theory was obtained following reversal of the macrophage's phenotype by either exogenous addition of IFN- α/β to GM-CSF-derived macrophages or by addition of anti-IFN- α/β antibodies to CSF-1-derived macrophages prior to VSV infection. These findings demonstrated clearly the role of IFN- α/β in differential viral susceptibility of these two macrophage populations. These findings support the hypothesis that there is a differential ability of GM-CSF- and CSF-1-derived macrophages to produce IFN- α/β endogenously. However, the mechanism by which VSV induced CPE in GM-CSF-derived macrophages is not known. Since neither GM-CSF- nor CSF-1-derived macrophages supported any significant viral replication (Table 4), it is unlikely that viral replication resulting in the release of infectious viral particles mediated the observed CPE in the GM-CSF-derived macrophage cultures. These findings are similar to those of McGowan and Wagner (1981) which demonstrated that virus production was not a prerequisite for CPE. In addition, observations that both populations of macrophages exhibited equal intensity of fluorescent staining of infected monolayers, as well as, equal numbers of infected cells (Figures 15 A,B,C,D) indicate that the CPE observed in GM-CSF-derived cultures may be attributable to differences in the maturation of viral progeny late in culture.

The mechanisms which underlie the apparent differences in the ability of these two CSFs to induce endogenous IFN production in macrophages is unclear. One possible explanation is that CSF-1 is a better inducer of endogenous IFN than GM-CSF. This theory was addressed directly by Lee and Warren (1987) by treating peritoneal macrophages with either CSF-1 or GM-CSF and measuring IFN production. These investigators confirmed previous observations that CSF-1 could induce IFN production (1987), but also demonstrated that mature macrophages treated with GM-CSF failed to produce IFN. An alternate explanation might be that GM-CSF blocks induction of endogenous IFN- α/β and/or precludes macrophages from

responding to exogenous addition of IFN- α/β . This latter suggestion is supported by the failure of IFN- α/β (50 U/ml) to increase Fc-mediated phagocytosis significantly in GM-CSF-derived macrophages. However, this anergy to exogenous IFN- α/β would have to be highly selective, since GM-CSF-derived macrophages responded to exogenous IFN- α/β in the generation of an antiviral state following treatment with as little as 1.0 U/ml. Thus, the failure of GM-CSF-derived macrophages to respond to exogenous IFN- α/β to exhibit increased Fc-mediated phagocytosis may reflect a defect in secondary signals required for Fc receptor induction which are not required for antiviral activity.

These findings may also indicate that IFN- α/β must be present during the macrophage differentiative process for full expression of Fc receptor capacity as well as a subsequent ability to respond to IFN- α/β . The role of IFN during differentiation in these bone marrow-derived macrophages will require further study. Inclusion of either IFN- α/β or antibodies to IFN- α/β during the differentiation in liquid culture of BMP in the presence of GM-CSF and CSF-1, respectively, may elucidate the role of endogenous IFN in the generation and subsequent IFN-responsiveness of these two populations with regard to Fc receptor expression.

Resident and peritoneal exudate macrophages from *Lps*ⁿ versus *Lps*^d mice have been shown to exhibit differences with regard to both Fc-mediated phagocytosis and viral resistance (Vogel and Rosenstreich, 1979; Vogel and Fertsch, 1987). Peritoneal macrophages from the *Lps*ⁿ mice exhibit higher levels of Fc-mediated phagocytosis and antiviral activity than macrophages derived from *Lps*^d mice. These differences could be reversed by treatment of the *Lps*ⁿ macrophages with antibodies to IFN- α/β (Vogel and Fertsch, 1984; Vogel and Fertsch, 1987).

In many ways, these findings are analogous to the differences described above for CSF-1-derived and GM-CSF-derived macrophages from C3H/HeJ mice, i.e., *Lps*ⁿ (C3H/OuJ) peritoneal macrophages are phenotypically more like CSF-1-derived macrophages, whereas *Lps*^d (C3H/HeJ) peritoneal macrophages are functionally similar to GM-CSF-derived

macrophages. One potential explanation for this interesting parallel could be that C3H/OuJ macrophages are more like CSF-1-derived macrophages due to a greater number of CSF-1-responsive progenitors than C3H/HeJ mice. Expansion of these CSF-1-responsive progenitors might result in a larger proportion of these types of macrophages in the periphery of C3H/OuJ mice compared to C3H/HeJ mice. This possibility is not likely, given the data presented in Figures 3 and 5. Since both strains of mice possess the same number of CSF-1-responsive progenitors in the bone marrow and spleen, the differences in macrophage phenotypes between C3H/HeJ and C3H/OuJ macrophages cannot be simply a reflection of available progenitors. Alternatively, the ability to stimulate the apparently equal numbers of CSF-1-responsive progenitors may differ in these two strains of mice. Since the inability of C3H/HeJ mice to respond to LPS is reflected in all cell types (reviewed in Vogel and Rosenstreich, 1981), including fibroblasts (Ryan and McAdam, 1977), *in vivo* stimulation by LPS may fail to induce CSF-1 production by fibroblasts (a major cellular source of CSF-1), and therefore, result in a diminution in the level of CSF-1 in the C3H/HeJ mouse. The consequence of this reduced CSF-1 production in the C3H/HeJ mouse would result in an inability to stimulate the available CSF-1-responsive progenitors, thus resulting in a paucity of mature CSF-1-derived macrophages in the periphery.

Aside from the direct effects of reduced CSF-1 production on bone marrow progenitors in the C3H/HeJ mouse, studies by Moore *et al.* (1980) suggest a second level at which CSF-1 concentrations might affect expansion of CSF-1-responsive progenitors. In these studies, Moore *et al.* showed that macrophages derived from bone marrow progenitors under the influence of CSF-1 or treatment of peritoneal exudate macrophages with CSF-1 resulted in an increase in the capacity of these macrophages to respond, *in vitro*, to low concentrations of LPS to produce IL 1 and IFN- α/β . If the Lps defect in the C3H/HeJ mouse resulted in diminished production of CSF-1, C3H/HeJ macrophages might be less "sensitized" to the stimulatory effects of LPS *in vivo*. In contrast, macrophages derived from Lpsⁿ mice might respond to CSF-1 to exhibit heightened sensitivity to endogenous LPS *in vivo* and thus, exhibit increased

production of IFN- α/β . This increase in IFN- α/β concentration could, in turn, potentiate the proliferative stimulus supplied by CSF-1 (Moore and Rouse, 1983) and result in greater expansion of the CSF-1-responsive progenitor population, as well as augmented Fc receptor expression and resistance to viral infection.

These studies demonstrate the differential ability of bone marrow progenitors stimulated by GM-CSF or CSF-1 to result in the maturation of macrophages with different capacities for IFN- α/β production. These studies also demonstrate the far-reaching effects of such a deficiency, i.e., decreased Fc receptor expression, as well as increased susceptibility to virus infection. These findings also illustrate the potential role of secondary mediators produced in response to CSFs, i.e., IFN- α/β , in the initiation and propagation of a complex cytokine cascade involved in the generation of macrophages.

Ia antigen expression. Another well-characterized marker of macrophage differentiation is the ability to express Class II MHC or Ia antigens (Beller and Ho, 1982). This marker has been associated with "higher order" macrophage functions (Beller, 1984). The expression of Ia antigens is essential for the appropriate presentation of antigen by antigen-presenting cells, such as macrophages, to antigen-specific T cells (Rosenthal and Shevach, 1973; Heber-Katz *et al.*, 1982; Beller, 1984; Unanue and Allen, 1987). Recently, much attention has focused on the mechanisms by which Ia antigen expression is regulated. Specifically, IFN- γ has been shown to be a principal lymphokine responsible for upregulating Ia antigen expression on macrophages (Steeg *et al.*, 1980; Birmingham *et al.*, 1982; King and Jones, 1983; Vogel *et al.*, 1983; Warren and Vogel, 1985a), as well as on other cell types (Koch *et al.*, 1984; Berrih, *et al.*, 1985; Groenewegen *et al.*, 1986; Ruff *et al.*, 1986). In some studies, enhanced expression of Ia antigen expression has also been correlated with increased Ia-dependent accessory functions (Steeg *et al.*, 1980; Tzechoval *et al.*, 1981; Birmingham *et al.*, 1982; Beller, 1984). These studies were carried out primarily on macrophage populations which express low levels of Ia in the absence of inducer. However, it has also been recognized

for some time that subpopulations of highly Ia-positive macrophages exist (Cowing *et al.*, 1978; Tzeheval *et al.*, 1981), even in athymic nude mice (Lu *et al.*, 1981), where the availability of endogenously-produced, T cell-derived IFN- γ to induce and/or maintain Ia expression would be markedly limited. This raises the possibility that other mechanisms may contribute to the development of certain populations of Ia positive macrophages within the body.

Although the CSFs, GM-CSF and CSF-1, are principally responsible for the differentiation of macrophages along the monocytic lineage (Metcalf, 1986), it is not clear whether these two cytokines act on discrete or overlapping populations of bone marrow progenitors (Metcalf and Burgess, 1982; Hogg, 1986) or whether the mature macrophages which arise under the influence of these distinct factors are functionally distinct populations (Bursuker and Goldman, 1982; Bursuker and Goldman, 1983; Hogg, 1986). Early studies by Lee and Wong (1980;1982) demonstrated that macrophages derived from bone marrow cells under the influence of crude preparations of fibroblast-derived CSF (i.e., shown by others to be principally CSF-1; Stanley and Heard, 1977) or a Colony Stimulating Factor found in endotoxin-stimulated lung conditioned medium (i.e., either a GM-CSF or a mixture of colony stimulating factors; Burgess *et al.*, 1977; Nicola and Metcalf, 1986) differed in their expression of Ia. Specifically, those cells grown in the crude CSF-1 preparation exhibited lower Ia antigen expression and "antigen-presenting" activity than cells grown in lung conditioned medium, but the expression of Ia on the CSF-1-derived cells could be augmented by exposure of the cells to crude lymphokine preparations. Similarly, Calamai *et al.* (1982) showed that bone marrow cells cultured in crude fibroblast supernatants gave rise to macrophages which expressed low levels of Ia, but could be induced to become Ia-positive after exposure to lymphokines. These authors raised the possibility that CSF may act to antagonize induction of Ia antigen expression.

Using recombinant and highly purified preparations of GM-CSF and CSF-1, we have confirmed and extended these findings. Specifically, macrophages derived under the influence of recombinant or purified, natural CSF-1 possess lower basal levels of Ia antigen

expression, express reduced levels of Ia-specific mRNA, and are significantly less capable of inducing T cell proliferation following antigen exposure when compared to rGM-CSF-derived macrophages. Although significant T cell proliferation was observed in macrophage cultures following exposure to antigen, the absence of data utilizing an irrelevant antigen precludes us from definitively stating that the data shown in Figure 20 were a result of antigen-specific T cell proliferation. Thus, rGM-CSF gives rise to a population of mature macrophages which is intrinsically more Ia-positive and more capable of Ia-related functions than CSF-1-derived macrophages. Since prostaglandins have been shown to be anti-proliferative to lymphocytes (Kuehl, 1977; Hoffeld *et al.*, 1980; Gerns, 1982; Morley, 1982) and since prostaglandins have been shown to down-regulate Ia antigen expression on macrophages (Snyder *et al.*, 1982; Tripp *et al.*, 1986), the potential role of PGE in the differences observed in basal Ia expression and function of these two populations was examined. The finding that rGM-CSF-derived macrophages produce approximately ten-fold greater levels of PGE than CSF-1-derived macrophages would argue against the possible role of PGE in the maintenance of low basal Ia expression in CSF-1-derived macrophages. In addition, our findings are in agreement with those of Vespa *et al.* (1987) in which PGE had no effect on the modulation of Ia expression in a cell line which expresses Ia antigen constitutively, while demonstrating its inhibitory effects on transient Ia expression in macrophages induced by lymphokines.

Another possible explanation for the differences in basal Ia expression exhibited by these two populations may exist in the observation that Ia expression has been shown previously to be under negative control by IFN- α/β (Ling *et al.*, 1985; Inaba *et al.*, 1986; Fertsch *et al.*, 1987). Our finding, that CSF-1-derived macrophages produce more IFN- α/β than GM-CSF-derived macrophages, is consistent with the possibility that decreased Ia antigen expression in CSF-1-derived macrophages is due to a negative regulatory influence exerted by endogenously-produced IFN- α/β . Thus, it is possible that endogenous IFN- α/β production plays a significant role in the maintenance of low Ia antigen expression in CSF-1-derived macrophages and/or the over expression of Ia antigen in rGM-CSF-derived macrophages.

Further examination of this possibility will require the exogenous addition of either IFN- α/β or use of anti-IFN- α/β antibodies during the development of these macrophages.

Although rIFN- γ was found to increase Ia expression on CSF-1-derived macrophages, the ability of IFN- γ to increase the capacity of these cells to drive antigen-specific T cell proliferation was only minimal (Figure 20). The ability of macrophages to function as "antigen-presenting" cells is a complex process which involves antigen uptake, antigen processing, presentation of antigen in the context of appropriate Ia antigen, as well as the presence of additional macrophage-derived signals (reviewed in Unanue *et al.*, 1984). The inability of increased total Ia expression in CSF-1-derived macrophages to result in a functional increase in T cell stimulation poses an interesting question concerning the relevance of total Ia expression. One possible explanation for this finding may be that the density of Ia antigen available to the antigen-primed T cells is critical for maximal stimulation and may suggest that a threshold level of Ia is required for T cell proliferation in the presence of antigen. It is clear from the fluorescent antibody analysis (Figure 19) that the density of Ia antigen on rIFN- γ -treated, CSF-1-derived macrophages still remains less than that seen in rGM-CSF-derived cells (medium- or rIFN- γ -treated), even though the total Ia (as measured in the ELISA assay) is equivalent and the number of Ia-positive macrophages is actually greater (as measured by direct cytotoxicity). Thus, the density of Ia on individual CSF-1-derived macrophages may simply be inadequate (i.e., below threshold levels) for optimal antigen presentation. Alternatively, reduced presentation function may reflect a failure of CSF-1-derived macrophages to process antigen correctly or to develop some additional cell surface marker(s) which is(are) necessary for T cell stimulation, such as Interleukin 1 (Kurt-Jones *et al.*, 1985; Virgin *et al.*, 1985; Unanue and Allen, 1986; Hurme, 1987; Morrissey *et al.*, 1987). While we have no data which compares the processing capabilities of these two populations of cells, the possible involvement of IL 1 in the differences observed with respect to T cell-induced proliferation has been examined. Preliminary studies showed that no detectable IL 1 was found in supernatants of medium-treated rGM-CSF- or CSF-1-derived

macrophages. However, the role of membrane-bound IL 1 may be of importance and requires further investigation (Kurt-Jones *et al.*, 1985; Virgin *et al.*, 1985; Gerrard *et al.*, 1987; Hurme, 1987).

Lastly, the finding that exposure of rGM-CSF-derived macrophages to high levels of rIFN- γ led to an inhibition of T cell proliferation at high cell concentration is consistent with previous reports of IFN- γ -induced suppression of antigen-stimulated lymphocyte proliferation (Wing *et al.*, 1986; Fujiwara *et al.*, 1987). Although the inhibitory factor in these preparations has not been identified definitively, one must examine the potential role of PGE in the inhibition of antigen-specific proliferation observed in rIFN- γ -treated GM-CSF-derived macrophages at high cell densities. Although additional mechanistic investigation is required, the findings reported herein establishes a functional distinction between macrophages derived under the influence of GM-CSF versus CSF-1. Since the production of GM-CSF has been ascribed to cell types other than T cells (Nicola *et al.*, 1979; Groopman, 1987; Chodakewitz *et al.*, 1988), it is possible that this form of CSF is responsible for the development of Ia positive macrophages in specific organs in the absence of a sustained IFN- γ signal.

Tumoricidal activity. The demonstration of high Ia expression in GM-CSF-derived macrophages prompted us to investigate an additional "higher order" differentiation function, that of acquisition of tumoricidal activity. The theory of a multisignal differentiation pathway which leads to the activation of macrophages to a fully tumoricidal state was originally put forth by Hibbs and his colleagues (Hibbs *et al.*, 1977; Weinberg *et al.*, 1978) and has since been confirmed and extended in many laboratories (Weinberg *et al.*, 1978; Pace and Russell, 1981; Pace *et al.*, 1983a,b; Schreiber *et al.*, 1983; Pace *et al.*, 1985a; Hogan and Vogel, 1987). Briefly, Hibbs *et al.* proposed that induction of maximal tumoricidal activity was a multi-signal event: macrophages were first "primed" into a stimulated, but non-cytolytic state by lymphokine(s), generically referred to as "MAF". Once "primed", the macrophage could be activated to full tumoricidal capacity by a "trigger" signal. Environmental stimuli, such as Gram negative LPS, were identified as potent "trigger" signals. Among the many early studies that addressed this

hypothesis experimentally, the work of Weinberg and his colleagues provided the important insight that the nature and potency of the "priming" signal defined the receptivity of a macrophage to a triggering signal (Weinberg *et al.*, 1978). For instance, macrophages derived from mice which had been administered a potent activating agent, such as Bacillus Calmette Guerin, required a very low dose of LPS to trigger tumoricidal activity. In contrast, if the macrophages were first elicited by a relatively weak stimulating agent, such as proteose peptone, either high concentrations of LPS or an additional lymphokine exposure followed by low doses of LPS, induced full tumoricidal activity.

Using the P815 mastocytoma as a target tumor cell, Pace *et al.* (1983a; 1983b) and Schreiber *et al.* (1983) defined IFN- γ as a principal form of Hibb's proposed MAF in lymphokine and T cell hybridoma supernatants. With the availability of recombinant IFN- γ , these observations were defined more precisely: maximal tumoricidal activity was induced in proteose peptone-elicited macrophages (derived from LPS-responsive C3H/HeN mice) with 1 - 10 U/ml rIFN- γ and as little as 0.3 ng/ml LPS. Neither rIFN- γ alone nor LPS alone induced any significant increase in tumoricidal activity in these macrophages. If macrophages were derived from the LPS-hyporesponsive C3H/HeJ strain, protein-free LPS could not be used as a "triggering" signal; however, alternate second signals, such as heat-killed *L. monocytogenes* (Pace *et al.*, 1985a), protein-rich But-LPS (Pace *et al.*, 1983b; Hogan and Vogel, 1987), or lipid A-associated protein itself (Hogan and Vogel, 1987), were fully efficacious "triggering" signals.

While much work has focused on rIFN- γ as MAF, work by Meltzer *et al.* (1982) laid the groundwork for an analysis of other cytokines for their ability to provide MAF-like priming signals. In their report, supernatants from the EL-4 thymoma cell line were shown to contain a 23,000 dalton protein which possessed MAF activity and co-eluted with CSF activity. These characteristics are consistent with a potential role for GM-CSF in this activation. The possibility that GM-CSF, a T cell-derived product (Ruscetti and Chervenick, 1975; Prystowsky *et al.*, 1983; Metcalf, 1986), could mediate these responses was addressed directly by Grabstein *et al.* (1986) in which human peripheral blood mononuclear cells were activated to a tumoricidal

state upon exposure to human recombinant GM-CSF, in the absence of exogenous LPS. However, data showing the effect of combined rGM-CSF and LPS were not provided in this report. The ability of fibroblast-derived CSF-1 to activate resident or elicited peritoneal macrophages has also been examined. Early work by Ampel *et al.* (1982) showed that treatment of peritoneal exudate cells with partially purified CSF-1 led to as much as an 80% inhibition of ^3H -thymidine uptake by target tumor cells. However, the media in this study was reported to contain up to 10 ng/ml LPS. In later studies, Wing *et al.* (1986) treated resident peritoneal macrophages with partially purified CSF-1 in media which contained < 0.1 ng/ml LPS. Under these conditions, the CSF-1 treatment failed to induce tumoricidal activity and the addition of LPS (1 ng/ml) increased activity to approximately 10%. This low level of induction was compared to approximately 34% in peritoneal macrophages treated with rIFN- γ (10.0 U/ml) and LPS.

In studies by Weinberg *et al.* (1978) and Keller and Keist (1986), crude CSF-1-containing supernatants were used to culture macrophages from peritoneal or bone marrow macrophage progenitors. In the Weinberg study, the CSF-1-derived macrophages failed to exhibit any tumoricidal activity unless treated with high doses of LPS. In the study by Keller and Keist (1986) the CSF-1-derived macrophages were non-tumoricidal, but could be fully activated by crude "MAF" preparations. The LPS content of these MAF preparations was not reported.

The data presented herein provide a side by side comparison of the tumoricidal capacities of macrophages derived from bone marrow progenitors under the influence of murine rGM-CSF or highly purified CSF-1. By using bone marrow progenitors from C3H/HeJ mice and assuring that all reagents were used at "endotoxin-free" concentrations (< 0.01 ng/ml), we were better able to compare the tumoricidal activities of macrophages following differentiation in rGM-CSF or CSF-1. Our data show that exposure of progenitors to rGM-CSF alone results in the development of a macrophage population which exhibits a higher basal level of cytotoxicity, respond slightly to a strong "priming" signal only, fail to respond to

"triggering" signal alone, and becomes highly tumoricidal with combined treatment. The basal cytolytic activity of these cells is consistent with the study of Grabstein *et al.*, (1986) which demonstrates that GM-CSF could activate mature human mononuclear cells to a tumoricidal state in the absence of exogenous LPS. Moreover, our finding that combined priming and triggering signals are synergistic with regard to tumoricidal activity indicate that macrophages derived under the influence of GM-CSF are not fully activated *per se*, but require two signals for maximal tumoricidal induction.

By comparison, CSF-1-derived macrophages exhibit no significant tumoricidal activity in the absence of exogenous signals. Treatment with rIFN- γ alone failed to increase this capacity significantly. However, the highest dose of But-LPS triggered significant tumoricidal activity, but only up to the basal levels exhibited by rGM-CSF-derived macrophages. Combined treatment of these cells also led to synergistic tumoricidal activity, but the maximal inducible level approached, but never attained, maximal levels attained in rGM-CSF-derived cultures.

The mechanism involved in the tumoricidal capacities of these two populations of macrophages remains to be determined. Given the mounting evidence that TNF as well as other monokines play a major role in macrophage-mediated tumoricidal activity (Jadus *et al.*, 1986; Philips and Epstein, 1986; Chen *et al.*, 1987; Ruggiero *et al.*, 1987), the potential role of differential TNF production between GM-CSF and CSF-1-derived macrophages must be investigated. Preliminary examination showed no statistical difference in the basal level and induced levels of TNF produced by these two macrophage populations (data not shown). However, recent reports indicate that membrane-bound TNF may play an important role in tumoricidal activity by macrophages (Decker *et al.*, 1987; Klostergaard *et al.*, 1987) and, therefore, should be examined in future studies.

Taken collectively, the studies presented herein have taken advantage of highly purified or recombinant CSFs to elucidate a mechanism(s) for the development of morphological and functional heterogeneity demonstrated within mature bone marrow-derived macrophages. Our findings support the theory that the macrophage

heterogeneity observed within specific organ systems may result from differential stimulation of heterogeneous populations of progenitors. The ability of GM-CSF to stimulate a limited number of progenitors in comparison to CSF-1 (Figure 3), as well as its ability to synergize with CSF-1 to expand proliferatively a subpopulation of HPP-CFC whose progeny are phenotypically more like GM-CSF- than CSF-1-derived macrophages (Table 11 and 12), suggest a role for GM-CSF as a potent regulatory cytokine in macrophage differentiation.

These findings extend a growing list of functional differences between macrophages derived under the influence of rGM-CSF versus CSF-1. Although CSF-1-derived macrophages are "more differentiated" with respect to their size, phagocytic capacities, and the ability to resist viral infection, rGM-CSF-derived macrophages exhibit a very high basal level of Ia antigen expression, an augmented capacity to induce antigen-specific T cell proliferation, as well as a greater potential for tumoricidal activity. The linear differentiation scheme which was originally put forth by Cohn (1978), and which has served as a "standard", implies that as a macrophage matures to its fully activated state, it must acquire certain markers and functions prior to the acquisition of "higher order" markers and functions. The findings presented herein suggest that under the influence of GM-CSF, the acquisition of "higher order" functions may not require a differentiative progression which results in the retention of certain "lower order" functions. In addition, our findings imply that functional heterogeneity among different populations of mature macrophages (Lee, 1980) may reflect the effects of different species of colony stimulating factor on progenitors and the emigration of selected populations to particular organs. For example, high Ia expression in macrophages in the spleens of euthymic (Cowing *et al.*, 1978) and athymic mice (Lu *et al.*, 1981; Wentworth and Ziegler, 1987), maintenance of high Ia expression in peritoneal macrophages derived from *B6g^F* mice (Zwilling *et al.*, 1987), differences in tumoricidal activation associated with different strains of mice (Pace *et al.*, 1985b), as well as constitutively high Ia expression on Langerhans cells (Tamaki *et al.*, 1979; Schuler *et al.*, 1985; Inaba *et al.*, 1986; Shimada *et al.*,

1987), may in part reflect the effect of GM-CSF versus CSF-1 on progenitors which then "home" to specific organs.

In conclusion, the demonstration of macrophages which possess such distinct phenotypes following expansion of BMP in a controlled environment, and following stimulation by a single exogenous CSF, has allowed for the definitive demonstration that preferential stimulation of BMP populations can result in the degree of heterogeneity previously observed in complex organ systems. The demonstration that GM-CSF-derived macrophages possess predominantly "higher order" differentiative functions while CSF-1-derived macrophages possess "lower order" functions calls into question the linear acquisition of macrophage differentiation markers previously reported. Table 13 provides a semi-quantitative summary of the data presented in this dissertation with respect to the functional and morphological differences observed in GM-CSF- and CSF-1-derived macrophages. Taken collectively, these data suggest that an alternate pathway of macrophage differentiation exists for macrophages derived under the influence of GM-CSF, i.e., that these cells by-pass acquisition of some of the early functional markers in their differentiation to the fully activated state (Figure 30). From a teleological standpoint, the demonstration of highly activated GM-CSF-derived macrophages is rather logical: given the low numbers of progenitors which respond to GM-CSF, and the fact that GM-CSF is primarily produced by activated T cells during the course of an inflammatory response, it is not surprising that the macrophages which develop from this limited number of progenitors are more highly activated, and thus, better suited for protecting an animal from an inflammatory insult.

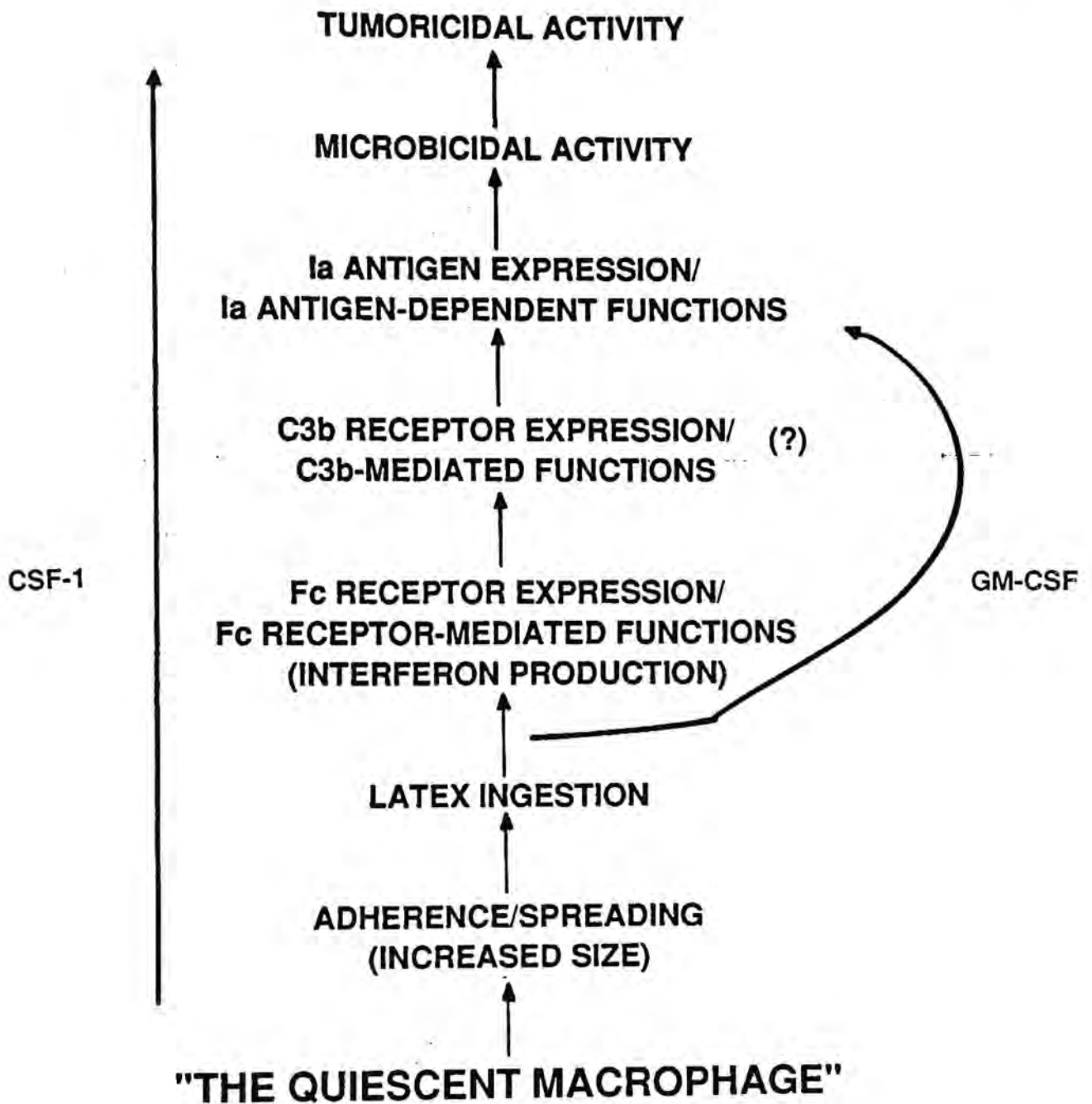
Table 13

Summary of Morphological and Functional Characteristics of GM-CSF- and
CSF-1-Derived Macrophages^a

<u>FUNCTIONS</u>	<u>GM-CSF-DERIVED</u>	<u>CSF-1-DERIVED</u>
1. Size/adherence	+	+++
2. Latex ingestion	+++	++++
3. Fc-receptor mediated ingestion	++	++++
4. Endogenous IFN production	-	++
5. Virus sensitivity	sensitive	resistant
6. Total Ia antigen expression		
medium:	++++	+
plus IFN- γ :	++++	+++
7. Stimulation of T cells in the presence of antigen		
medium:	++++	++
plus IFN- γ :	++++	+++
8. Tumoricidal activity		
medium:	+	-
plus IFN- γ and But-LPS:	++++	++ to +++

^a This qualitative summary was based upon the quantitative data presented throughout the Results section of this dissertation.

Figure 30. Modified pathway of macrophage differentiation based on the work presented in this dissertation. The hierarchy of macrophage differentiation shown in Figure 1 has been modified to incorporate a potential alternate pathway by which GM-CSF-derived macrophages proceed differentially.



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